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FACULDADE DE MEDICINA

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Tese orientada pelo Professor Doutor Luis Filipe Ferreira Moita

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Resumo

Palavras-chave: "Splicing" Alternativo, Inflamação, Interleucina-1β, Factores de "Splicing"

A descrição dos quatro sinais de Inflamação (*rubor, calor, tumor e dor*) por *Celsus* retrata talvez pela primeira vez a existência desta resposta Imune (revisto por *Rocha e Silva,* 1978). Durante muito tempo, como descrito por *Metchnikoff* em 1892, julgava-se necessária a presença de um micróbio para a indução de Inflamação, no entanto, sabe-se actualmente que a sua presença não é necessária para que o processo de Inflamação ocorra, sendo cada vez mais observadas respostas inflamatórias na sua ausência (revisto por *Metchnikoff,* 1892; *Dinarello,* 2004; *Medzhitov,* 2008). Ao longo dos últimos anos, têm surgido novas áreas de investigação com a finalidade de estudar o complexo mecanismo de regulação da resposta inflamatória. Alguns dos grandes marcos que originaram mudanças significativas na compreensão do mecanismo de Inflamação foram (1) a descoberta da secreção de mediadores pró-inflamatórios por parte das células do Sistema Imune (como por exemplo, as Interleucinas 1β (IL-1β) e 8 (IL-8), *Menkin*, 1944; *Dinarello,* 2009), (2) a definição de "Pattern Recognition Receptors" (*Janeway,* 1989), como receptores envolvidos no reconhecimento de diversas estruturas conservadas presentes em diversos micróbios ou mediadores de sinalização celular; e (3) a descoberta do Inflamassoma, um complexo multiproteico envolvido no processamento de diversas Interleucinas, incluindo a IL-1β (*Martinon et al.,* 2002). Com a melhoria dos conhecimentos relativos ao mecanismo de regulação da resposta inflamatória, foi possível associar mutações em componentes-chave desta resposta a um elevado número de doenças, como é o

caso do Síndrome de Muckle-Wells ou a Febre Mediterrânica Familiar, mutações ao nível do NALP3 ou Pyrin, respectivamente *(Ogura et al.,* 2001; *Maeda et al.,* 2005; *McGonagle et al.* 2007; *Church et al.* 2008; *Martinon et al.,* 2009).

No que respeita às citocinas pró-inflamatórias, a IL-1β foi uma das primeiras Interleucinas a ser identificada e representa um dos mais importantes mediadores da Inflamação e da resposta do hospedeiro à infecção (revisto por *Dinarello,* 2004). A elevada correlação entre defeitos na regulação da secreção desta citocina e o aparecimento de doenças inflamatórias, fez desta um dos focos de maior investigação na área da Inflamação nos últimos anos (revisto por *O'Neill,* 2008). Actualmente, várias doenças são já tratadas com sucesso, quer com antagonistas do receptor de IL-1β, como por exemplo usando o antagonista Anankira, quer com outros métodos que visam diminuir a concentração de IL-1β, recorrendo, por exemplo, à neutralização de IL-1β com o recurso a anticorpos monoclonais anti-IL-1β (revisto em *Fitzgerald et al .,* 2005; *Kalliolias et al.,* 2008; *Lequerre et al.* 2008; *Martinon et al.,* 2009).

O processo que leva à secreção da IL-1β ocorre essencialmente em dois passos fundamentais, a Produção e o Processamento. Esta citocina é produzida numa pró-forma inactiva (pro-IL-1β), sendo maioritariamente transcrita após a activação do factor de transcrição "Nuclear Factor-kB" (NF-kB) e seguidamente traduzida em proteína. Posteriormente, esta forma inactiva de IL-1β é processada na sua forma activa, por um mecanismo que envolve a presença da Caspase-1, num passo que precede a sua secreção (revisto por *Martinon et al.,* 2009). Por sua vez, a Caspase-1 é também sintetizada de forma inactiva, que requer processamento pelo complexo multiproteico Inflamassoma para se tornar activa (revisto por *Martinon et al.,* 2009). Em suma, os dois passos que levam à secrecão da IL-1β são a Produção e a Activação do Inflamassoma, que levará à activação da Caspase-1, processamento da IL-1β e subsequente secreção desta. Porém, embora o complexo mecanismo envolvido na regulação da secreção desta citocina tenha vindo a ser

amplamente estudado, não é ainda inteiramente conhecido, pelo que é de extrema importância o seu estudo de uma forma mais aprofundada.

Com o aumento do conhecimento dos mecanismos envolvidos na regulação da secreção de IL-1β, alguns investigadores têm direccionado agora a sua atenção na pesquisa de como serão reguladas as moléculas envolvidas nesse processo, no decurso de uma resposta inflamatória. Foram identificados diversos níveis de mecanismos de regulação como: diferenças ao nível de expressão, distintos estados de fosforilação, existência de várias isoformas da mesma molécula, entre outros (revisto por *Hayden et al.*, 2004; *Lynch*, 2004; *Anderson*, 2010). Foram, também, identificados genes envolvidos na regulação da secreção de IL-1β como sendo regulados por "Splicing" Alternativo (AS), como por exemplo o MyD88, o IRAK2 e o NOD2 (revisto em *Leeman et al.,* 2008). Assim, o aumento do número de funções celulares regulados por AS bem como a forte correlação entre alterações ao nível do "Splicing" e a existência de doença (revisto por *Leeman et al.,* 2008; *Tazi et al.* 2008), indica que o AS pode desempenhar um papel importante na iniciação e na regulação de uma resposta inflamatória, particularmente na secreção de citocinas pró-inflamatórias como a IL-1β.

Na tentativa de identificar os Factores de "Splicing" (SF) que desempenhem um papel na secreção de IL-1β após um estímulo inflamatório, usamos como modelo o estímulo de uma linha celular monocítica (THP-1) com *E.coli*, na triagem da secreção daquela citocina recorrendo à tecnologia de RNA de interferência (RNAi). Resumidamente, foi utilizada uma sub-colecção da biblioteca de RNAi disponibilizada pelo "The RNAi Consortium" (TRC) que permitiu estudar os efeitos da perda de função de 425 genes envolvidos no mecanismo de "Splicing" durante a produção e secreção de IL-1β. Após o "knockdown" e posterior estimulação com *E.coli*, os níveis de IL-1β secretados foram medidos por ELISA. Após triagem e sucessivas experiências de validação, foram identificados 19 genes que afectam significativamente a secreção de IL-1β

no nosso modelo. Entre os candidatos validados, os factores de Splicing (SF), ASF/SF2 e SRp20 mostraram um fenótipo consistente com o de reguladores negativos, pois após a diminuição da sua expressão usando a técnica de RNAi, detectaram-se níveis elevados de secreção de IL-1β. Seguidamente, reverteu-se o fenótipo observado, por clonagem dos dois SFs e sobre-expressão na mesma linha celular. A sobre-expressão destes SF fez diminuir os níveis de IL-1β secretados, validando os resultados anteriores, e implicando o ASF/SF2 e o SRp20 como reguladores negativos da secreção de IL-1β.

Com o aumento de doenças associadas a alterações ao nível de "Splicing", nalguns estudos são usadas actualmente drogas que controlam a expressão ou actividade de diversos SF, de forma a modular as respostas em questão. Dois desses estudos, efectuados por *Stoilov* e *Keriel (Stoilov et al.* 2008; *Keriel et al.,* 2009), identificaram vários compostos capazes de modular eventos de "Splicing" dependentes destes dois SF em estudo. Usando estas mesmas drogas, observou-se um aumento na secreção de IL-1β após o estímulo com *E.coli*, uma vez mais confirmando os nossos resultados de RNAi.

A fim de avaliar o papel dos dois candidatos, ASF/SF2 e SRp20, nos dois passos necessários para a secreção de IL-1β, Produção e Processamento, foram efectuadas várias experiências. Analizaram-se os níveis de expressão de IL-1β por PCR quantitativo após o silenciamento destes dois candidatos e posterior estímulo com *E.coli*. Foram observados valores de expressão de IL-1β superiores nas células onde a expressão dos dois SF tinha sido anteriormente reduzida por RNAi. Os resultados permitem concluir que ambos os candidatos são reguladores negativos da produção de IL-1β. Como forma de verificar o impacto do "knockdown" dos dois candidatos no segundo passo necessário para a secreção de IL-1β, medimos a actividade da Caspase-1 recorrendo a uma técnica de citometria de fluxo (FACS). O "knockdown" do ASF/SF2 não apresentou qualquer impacto na activação de Caspase-1. Em oposição, observou-se um notável aumento de

activação de Caspase-1 após o "knockdown" do SRp20. Com estes resultados pudemos concluir que o SRp20 é um regulador negativo de ambas as etapas necessárias para a secreção de IL-1β, Produção e Processamento, enquanto que o ASF/SF2 é um regulador negativo da produção de IL-1β. Observaram-se também, níveis elevados de expressão de Caspase-1 após o "knockdown" do SRp20, podendo talvez desta forma explicar os níveis superiores de activação de Caspase-1 observados nestas condições.

Entre as diversas funções já descritas como sendo mediadas por estes dois SF, um estudo recente implicou o ASF/SF2 no processo inflamatório *(Xiong,* 2006). Nesse estudo foi demonstrado que a expressão do ASF/SF2 é reduzida em músculo inflamado ou após um estímulo inflamatório (por exemplo usando TNFα, *Xiong,* 2006). Decidimos então analisar a expressão dos dois candidatos mediante estimulação com *E.coli,* no sistema em estudo. Notou-se uma redução da expressão do ASF/SF2 ao nível do mRNA, ao passo que a expressão diminuída do SRp20 foi observada somente ao nível de proteína. Os mecanismos envolvidos neste processo de regulação dos dois SF mediante estimulação com *E.coli* estão agora a ser investigados.

Como supracitado, o aumento do número de genes que são regulados por AS que desempenham um papel na Inflamação precisam ser estudados mais profundamente. Foram inicialmente analisados os perfis de "Splicing" de alguns dos genes anteriormente identificados como sendo regulados por AS e que podem ter um papel activo na regulação da secreção de IL-1β. Foram estudados os padrões de "Splicing" de alguns genes após o "knockdown" do ASF/SF2 ou SRp20 e/ou estimulação com *E.coli*, porém sem resultados conclusivos. Assim, decidimos realizar uma análise das alterações nos padrões de "Splicing" recorrendo à tecnologia de "microarrays" usando a plataforma Affymetrix® GeneChip ® Human Exon 1.0 ST. Foram analisadas as alterações dos padrões de "Splicing" de todos os genes Humanos após "knockdown" do ASF/SF2 e/ou

estimulação com *E.coli*, ficando o estudo das alterações relativas ao outro candidato, SRp20, para futuras experiências. Após a análise dos dados e respectiva validação, vários dos genes, já anteriormente relatados em diversas publicações como sendo regulados por AS nas mesmas condições, foram encontrados utilizando a nossa abordagem, validando assim o nosso método. No futuro serão efectuados estudos mais detalhados para os genes mais promissores com o intuito de desvendar o seu papel na regulação da secreção de IL-1β.

Podemos concluir que a tecnologia de RNAi é uma ferramenta poderosa para desvendar os genes envolvidos em diferentes vias celulares. No nosso estudo, foram identificados vários SF que podem desempenhar um papel na regulação da secreção de IL-1β após um estímulo com *E.coli.* Entre os candidatos identificados, o ASF/SF2 foi demonstrado como desempenhando papel na primeira etapa da secreção de IL-1β, a Produção; ao passo que o outro candidato estudado, o SRp20, desempenha um papel importante, quer na Produção de IL-1β, quer na etapa de Processamento por parte do Inflamassoma. São necessários vários estudos para determinar os mecanismos pelos quais estes SF desempenham um papel regulador da secreção de IL-1β.

Abstract

Keywords: Alternative Splicing, Inflammation, Interleukin-1β, Splicing Regulation

Inflammation was one of the first immune responses to be reported to exist and has been a focus of intensive research ever since (reviewed in *Rocha e Silva*, 1978; *Medzhitov*, 2010). During the course of an inflammatory response several components are involved, such as inflammatory inducers and mediators or different sensors that mediate the detection of a pro-inflammatory stimulus. Amongst the most studied pro-inflammatory mediators is the Interleukin-1β (IL-1β). Interleukin-1β was one of the first Interleukins to be identified and represents one of the most important mediators of Inflammation and host responses to infection (reviewed in *Dinarello*, 2004). The large connection between misregulation of IL-1β release and the appearance of inflammatory diseases made this cytokine one of the "hotspots" of intensive research in the past years (reviewed in *O'Neill*, 2008). Nowadays, several diseases are being treated successfully using different methods that decrease IL-1β circulant levels or block its effects, such as using IL-1β receptor antagonists or by neutralizing IL-1β with monoclonal anti-IL-1β antibodies (reviewed in *Fitzgerald et al.*, 2005; *Kalliolias et al.*, 2008; *Lequerre et al.*, 2008; *Martinon et al.*, 2009).

The IL-1β secretion pathway is a "two-step" fashion mechanism, Production and Processing steps. The cytokine is produced in an inactive pro-form (pro-IL-1β) mainly upon activation of the nuclear factor-kB (NF-kB) transcription factor and posterior translation into protein. The second and processing step is mediated by active Caspase-1, that will give rise to its mature and secreted form (reviewed in *Martinon et al.*, 2009). Caspase-1 is also synthesized as an inactive

form that requires processing by the Inflammasome complex to become active (reviewed in *Martinon et al.*, 2009). Upon Inflammasome formation, Caspase-1 becomes active, leading to subsequent IL-1β processing and posterior release. However, despite the increase knowledge that has been obtained during the past years, a lot still remains to be unveiled concerning the mechanisms involved in the regulation of IL-1β secretion and the effects elicited by this cytokine.

Several genes involved in the regulation of IL-1β secretion were shown to be regulated by Alternative Splicing (AS), such as MyD88, IRAK2 and NOD2, among others (reviewed in *Leeman et al.*, 2008). Moreover, the increasing number of cellular processes regulated by AS and the strong correlation between defects in Splicing and disease (reviewed in *Leeman et al.*, 2008; *Tazi et al.*, 2008), strongly suggest that AS may play a role in the initiation and regulation of an inflammatory response, particularly in the secretion of the pro-inflammatory cytokine IL-1β.

In an attempt to identify the Splicing Factors and Regulators (SF) with a potential role in the secretion of IL-1β upon an inflammatory stimulus, we performed an RNAi-based screen. Briefly, we used a subset of the TRC Lentiviral Human Library to generate loss-of-function phenotypes for most of SFs. We silenced the expression of 425 genes involved in Splicing with an average 5-fold coverage. After the primary screen and several rounds of phenotypic validation, 19 genes were identified to significantly affect the secretion of IL-1β by THP-1 cells after a 24 hours challenge with *E. coli*, as measured by ELISA. Among the candidates, ASF/SF2 and SRp20, showed a clear negative regulator phenotype, where upon decreased expression of these two candidates, elevated levels of secreted IL-1β secretion were detected, in comparison to control. Thus, we decided to focus our attention in these two SFs. In order to confirm the phenotype observed using the RNAi technology, we overexpressed these two candidates in THP-1 cells. As expected, overexpression decreased the levels of secreted IL-1β, therefore validating the previous results as implicating ASF/SF2 and SRp20 as negative

regulators of IL-1β secretion. In addition, using drugs already described to block the Splicing dependant on ASF/SF2 or SRp20, we observed increased levels of IL-1β upon treatment, therefore, once more confirming the results obtained in the RNAi based screen.

Next, we studied the role of ASF/SF2 and SRp20 in the regulation of the two-steps necessary for IL-1β secretion. The levels of IL-1β mRNA expression were increased upon ASF/SF2 or SRp20 knockdown, when compared to control cells. We could then conclude that both SFs are negative regulators of IL-1β production. As mentioned before, the second step necessary for IL-1β secretion is its processing in a Caspase-1 dependant manner. We measured Caspase-1 activation by FACS in cells upon ASF/SF2 or SRp20 knockdown and posterior challenge with *E.coli*. Knocking-down ASF/SF2 did not show any impact in Caspase-1 activation. In opposition, a remarkable increase in Caspase-1 activation was observed upon SRp20 knockdown, as compared to control cells. Thus, we could conclude that SRp20 acts as a negative regulator of both IL-1β Production and Processing, whereas ASF/SF2 is a negative regulator of IL-1β Production. In addition, increased Caspase-1 mRNA expression was observed in SRp20 knockdown cells as compared to control, consequently suggesting that increased Caspase-1 activation in SRp20 knockdown cells might be due to increased Caspase-1 expression. However further experiments are still required to prove this assumption.

In the past years, several reports clearly show the involvement of these two SFs in the regulation of different cellular processes such as transcription, translation and apoptosis (reviewed in *Huang et al.*, 2004; *Li et al.*, 2005a*; Long et al.*, 2009). A recent report implicates ASF/SF2 in Inflammation (*Xiong*, 2006), as it was shown that ASF/SF2 is downregulated in inflamed muscle or after an inflammatory stimulus, such as TNFα. Thus, we decided to check the expression of ASF/SF2 and SRp20 upon *E.coli* challenge. Decreased expression of ASF/SF2

was observed at the mRNA level, whereas decreased expression of SRp20 was only observed at the protein level after an *E.coli* challenge. The mechanisms involved in the downregulation of these two SFs upon *E.coli* challenge are now being investigated.

The increased number of genes that are regulated by AS already reported to play a role in Inflammation strongly suggested us to look for their Splicing profiles in ASF/SF2 or SRp20 knockdown cells upon an *E.coli* challenge. Several PCR experiments were performed to identify differences in the Splicing patterns of the described genes, however with no conclusive results. Consequently, we decided to perform an unbiased and high-throughput analysis of Alternative Splicing Events (ASE) that take place either after an *E.coli* challenge or after knockdown of ASF/SF2, using the Affymetrix® GeneChip® Human Exon 1.0 ST Arrays platform. Upon data analysis and validation, several genes already reported to undergo AS in different publications were found using our approach, therefore validating our method. We are now focusing on the most relevant candidates to perform follow-up studies.

In sum, this work allowed us to identify two novel regulators of IL-1β secretion after an *E.coli* challenge. Our results clearly show that both ASF/SF2 and SRp20 are negative regulators of IL-1β secretion. While SRp20 is involved in the regulation of the two-steps required of IL-1β secretion, Production and Processing, ASF/SF2 is only involved in the regulation of the first step, Production. In addition to IL-1β, increased Caspase-1 expression was also observed upon SRp20 knockdown, suggesting the involvement of this SF in the regulation of the expression of this inflammatory Caspase. Moreover, the expression of the two SFs was also shown to be altered in the course of an inflammatory response to *E.coli* challenge, however the mechanism involved has yet to be determined. Several studies are now required to determine the mechanisms by which these two SFs play a role in IL-1β secretion.

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This thesis marks definitely a “turning-point” in my life. To be a researcher has always been one of my main goals, and once I was given the opportunity to do a PhD I did not think twice before entering this challenge. Without the help of several people I could have never been sucessfull and overcome the major obstacles that I had to face during this long journey.

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Preface

This thesis presents the results of the research that was carried out at the Institute of Molecular Medicine, Faculty of Medicine, University of Lisbon, between 2007 and 2009 under the supervision of Professor Luis Filipe Ferreira Moita.

The main goal of this work was to identify Splicing Factors that play a role in the regulation of the pro-inflammatory cytokine, Interleukin-1β, secretion. During the course of this work we have identified several Splicing factors showing an impact in Interleukin-1β secretion after *E.coli* challenge. Two of the Splicing Factors were studied in greater detail to identify their possible mechanisms of regulation. The work present in this thesis is currently in preparation for submission for publication in the near future.

During the course of my PhD, I have also performed other studies. Two of the studies that I have performed in collaboration are now being submitted or accepted for publication:

Mishra, B.B. *, **Moura-Alves**, P.*, Sonawane, A., Hacohen, N., Griffiths, G., Moita, L.F., Anes, E. *"The Mycobacterium tuberculosis Protein ESAT-6 is a Potent Activator of the NLRP3/ASC Inflammasome". 2010. Cellular Microbiology* (accepted for publication).
* Contributed equally to the work

Casalou, C., **Moura-Alves, P.,** Carvalho, T., Beira, J., Caiado, F., Moita, L.F., Dias, S., *"MNK-1 kinase regulates acute leukemia VEGF production"* (in preparation).

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List of Abbreviations

AD - Activation Domain

AP-1 - Activator Protein-1

AS - Alternative Splicing

ASC - Apoptosis-associated Speck-like Protein

ASE - Alternative Splicing Events

ATP - Adenosine Triphosphate

BIR - Baculovirus IAP Repeat

CARD - Caspase Recruitment Domain

cDNA - complementary DNA

CIITA - MHC class II Transcription Activator

CINCA - Chronic Infantile Neurologucal Cutaneous and Auricular Syndrome

COP - CARD Only Protein

DAMP - Damage Associated Molecular Pattern

DC - Dendritic Cell

DEG - Differentially Expressed Genes

DNA - Deoxyribonucleic Acid

dsDNA/RNA - Double stranded DNA/RNA

E.coli - Escherichia coli

ELISA - Enzyme-linked Immunosorbent Assay

ERK - Extracellular-signal Related Kinases

ESE - Exonic Splicing Enhancer

ESS - Exonic Splicing Silencer

FACS - Fluorescent Activated Cell Sorting

FIIND - Function to Find

FMF - Familial Mediterranean Fever

GAPDH - Glyceraldehyde-3-Phosphate Dehydrogenase

HMGB1 - High Mobility Group Box 1

hnRNP - heterogenous nuclear Ribonucleoproteins

HPF - Hereditary Periodic Fever

IFN - Interferon

IKK - Inhibitor of Nuclear Factor-kB(IkB) Kinase Complex

IL-18 - Interleukin 18

IL-1β - Interleukin 1 beta

IL-1R - Interleukin 1 Receptor

IL-8 - Interleukin 8

IRAK - IL-1R Associated Kinase

ISE - Intronic Splicing Enhancer

ISS - Intronic Splicing Silencer

ITAM - Immunoreceptor Tyrosine based Activation Motif

JNK - c-JUN N-Terminal Kinases

LPS - Lipopolysaccharide

LRR - Leucine Rich Repeat

MAPK - Mitogen Activated Protein Kinase

MBL - Mannan Binding LEctin

MDA-5 - Melanoma Differentiation Associated Gene 5

MDP - Muramyl Dipeptide

miRNA - micro RNA

mRNA - messenger Ribonucleic Acid

MWS - Muckle Wells Syndrome

MyD88 - Myeloid Differentiation Primary Response Gene 88

M.tb - Mycobacterium tuberculosis

NF-kB - Nuclear Factor-kB

NLR - NOD-like Receptor

XXX

NOD - Nucleotide Oligomerization Domain

PAMP - Pathogen Associated Molecular Pattern

PCR - Polymerase Chain Reaction

PFA - Paraformaldehyde

PGN - Peptidoglycan

PMA - Phorbol 12-Myristate 13-Acetate

POP - Pyrin Only Protein

pre-mRNA - precursor messenger RNA

PRR - Pattern Recognition Receptor

PYD - Pyrin Domain

qRT-PCR - quantitative Real Time PCR

RIG-I - Retinoic Acid Inducible Gene 1

RISC - RNA Induced Silencing Complex

RLR - RIG-like Receptor

RNA - Ribonucleic Acid

RNAi - RNA interference

RRM - RNA Recognition Motif

RRMH - RRM Homology

SF - Splicing Factor

shRNA - short hairpin RNA

siRNA - short interference RNA

snRNP - small nuclear Ribonucleoprotein

SR - Serine/Arginine-rich

ssDNA/RNA - single stranded DNA/RNA

TAB - TAK Binding Protein

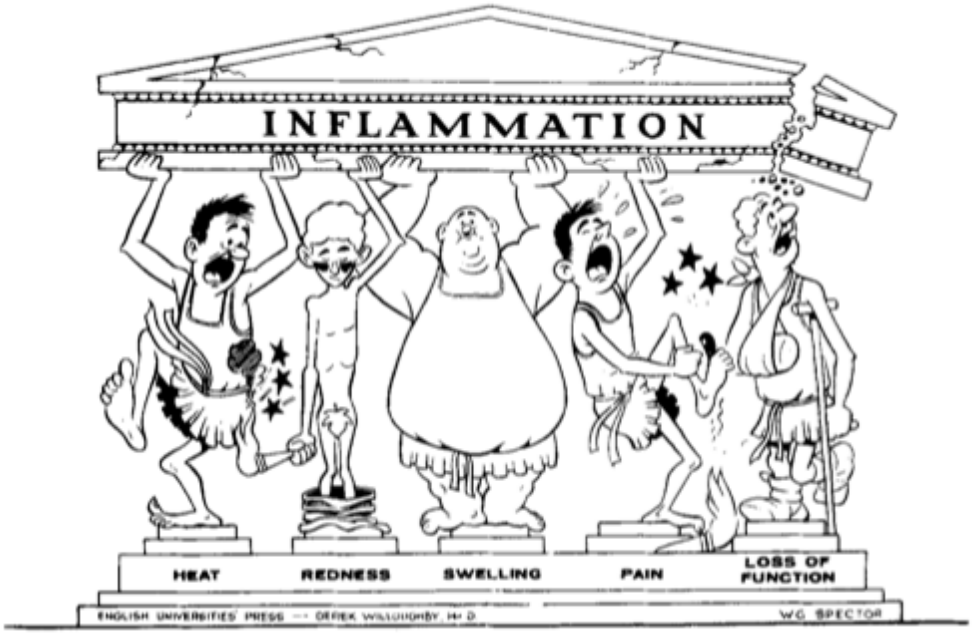
TIR - Toll/IL-1R Homology Domain

TLR - Toll-like Receptor

TNF - Tumour Necrosis Factor

TRAF6 - Tumour Necrosis Factor Associated Factor 6

Chapter 1 - Introduction



The four signs of Inflammation by Celsus: calor, rubor, tumour and dolor.

(Adapted from Rocha e Silva, 1994).

Inflammation is normally defined as a complex response to infection, trauma and other conditions of homeostatic imbalance (reviewed in *Nathan*, 2002). If one wants to trace the first descriptions of the Inflammatory response, we would probably have to go back to beginning of the first century A.D., where *Celsus* described the four signs of Inflammation: *rubor* (redness), *tumour* (swelling), *calor* (heat) and *dolor* (pain) (reviewed in *Rocha e Silva*, 1978). Several other descriptions of this process can be found along the years, such as *Galen*'s definition in the third century A.D, as the "reaction of the body against injury" (reviewed in *Rocha e Silva*, 1978). However, some authors claim that the real history of Inflammation started in the middle of the nineteenth century, when scientific methods began to be applied in higher extent to unveil the causes of many infections by germs and viruses. More than 100 years ago, *Metchinikov* described that an Inflammatory response is normally mounted as a response to a pathogenic microbe (*Metchnikoff*, 1892), however, the presence of an infectious agent is not always mandatory to induce Inflammation, as we know nowadays (reviewed in *Medzhitov*, 2008).

The Inflammatory response to infection consists of multiple components with distinct functions. Along the past years, several new "highways" of research have been "opened", namely after the discovery of the release of pro-inflammatory mediators by Immune cells (such as the cytokines, *Menkin*, 1944; *Dinarello*, 2009), the notion of Immune Pattern Recognition Receptors (PRR) by Janeway's (*Janeway*, 1989) and more recently with the discovery of the Inflammasome, as an important multicomponent complex that regulates the secretion of several cytokines, such as the Interleukin-1β (IL-1β, *Martinon et al.*, 2002). Upon recognition of a specific molecular signature, normally defined as a Pathogen Associated Molecular Pattern (PAMP) by the PRR, the Inflammation signaling cascade is activated and needs to be tightly regulated, otherwise the excessive Inflammatory process can lead to a variety of pathological conditions, including

septic shock, autoimmunity, as other chronic Inflammatory disorders (*Karin et al.*, 2006; *Church et al.*, 2008; *Medzhitov*, 2008; *Martinon et al.*, 2009). Thus, a defect in the PRR signaling pathway makes the host susceptible to various pathogens, and the overactivation or misregulation can lead to a chronic Inflammatory disease (reviewed in *Medzhitov*, 2008).

A growing number of systemic Inflammatory diseases, characterized by fever, anemia, and elevated levels of acute-phase proteins, have been linked to excessive production and elevated circulating levels of IL-1β (reviewed in *Dinarello*, 2009). Thus, seminal work has been performed in the past years attempting to better understand the underlying mechanisms involved in the regulation of this important pro-inflammatory cytokine. The mechanism of IL-1β secretion has been extensively studied, and made this field as one of the "hotspots" in the last years. Important regulators have been found to be able to modulate this response in order not to be detrimental to the body, however the entire picture is not fully drawn so far. Different regulator molecules were found to be expressed upon the initiation of the Inflammatory process, such as Decoy Receptors, that show the capability to modulate the extent of an Inflammatory response (reviewed in *Liew et al.*, 2005; Lee et al., 2007; Martinon et al., 2009).

Nowadays, due to the actual knowledge related to the regulation of the secretion of this cytokine, several patients are being treated successfully using IL-1β receptor antagonists, such as Anankira, or other methods to decrease IL-1β concentrations, for example neutralizing IL-1β by monoclonal anti-IL-1β antibodies (reviewed in *Fitzgerald et al.*, 2005; *Kalliolias et al.*, 2008; *Lequerre et al.*, 2008; *Martinon et al.*, 2009).

However, despite the intensive work that have been developed in the past years, the entire mechanism involved in the regulation of IL-1β secretion is not yet fully unveiled. Thus, we have decided to study the regulation of IL-1β secretion in the context of a bacterial challenge, such as *Escherichia coli (E.coli)*.

Among the mechanisms already found to regulate IL-1β secretion, it was found that several genes involved in this pathway are regulated post-transcriptionally by an Alternative RNA Splicing (AS) dependant mechanism (reviewed in *Wells et al.*, 2006; *Leeman et al.*, 2008). Thus, the increasing number of genes and cellular processes found to be regulated by AS (*Pan et al.*, 2008; *Wang et al.*, 2008), and the strong connection between AS misregulation and disease (reviewed in *Leeman et al.*, 2008; *Tazi et al.*, 2008), lead us to study the role that it may play in the initiation and regulation of an Inflammatory response, particularly in the secretion of the pro-inflammatory cytokine IL-1β. We decided to study the impact of different AS regulators in the context of IL-1β secretion regulation.

Chapter 1.1 - Interleukin-1β and Inflammation

"The experiment I have just outlined shows the first stage of Inflammation in the animal world. Now Inflammation as understood in man and the higher animals is a phenomenon that almost always results from the intervention of some pathogenic microbe. So it is held that the afflux of mobile cells towards points of lesion shows the organism's reaction against foreign bodies in general and against infectious microbes in particular. On this hypothesis, disease would be a fight between the morbid agent, the microbe from outside, and the mobile cells of the organism itself. Cure would come from the victory of the cells and immunity would be the sign of their acting sufficiently to prevent the microbial onslaught."

Metchnikoff, Nobel Lecture in 1908

Inflammation is one of the oldest Immune responses reported to occur, where the description of the four signs of Inflammation by *Celsus* (*rubor*, *tumour*, *calor* and *dolor*) in the I century A.D., represents probably the first "building-stone" (reviewed in *Rocha e Silva*, 1978). Along the years, the notion of Inflammation as an Immune response triggered by tissue injury, with the goal to restore organism's homeostasis became clear, as can be observed in the definitions by *Galen* (III century A.D) and *Neumann* (1889), "a reaction of the body against injury" or as "a series of local phenomena developing as a result of primary lesions to the tissues and that tend to restore their health", respectively (reviewed in *Rocha e Silva*, 1978). In the XIX century, the work of *Elie Metchnikoff*, clearly marks the importance of Inflammation as an Immune system reaction to the presence of

infectious microbes, preventing the organism against disease (*Metchnikoff*, 1892). However, as we know nowadays, despite the great importance of this Immune response against infection, the presence of a microbe is not mandatory to an Inflammatory response to occur (reviewed in *Medzhitov*, 2008).

The Inflammatory response is characterized by an increased blood flow and vascular permeability along with the accumulation of fluid, leukocytes, and the release of Inflammatory mediators. Among the released mediators of Inflammation is a group of cell-derived polypeptides, known as cytokines, which play an important role in the outcome of this response (reviewed in *Oppenheim*, 2001). The different roles played by these molecules in the generation and regulation of this Immune response definitely shows their importance as large "orchestrators" of Inflammation (reviewed in *Feghali et al.*, 1997; *Oppenheim*, 2001). The first description of cytokine dates from *Menkin's* work that "purified" factors ("pyrexins") with fever-inducing capacities from Inflammatory exudates (*Menkin*, 1943; *Menkin*, 1944).

The Inflammatory cytokines can be divided into different families according to their pro or anti-inflammatory properties, structural similarities or their involvement in acute or chronic Inflammation (reviewed in *Feghali et al.*, 1997; *Dinarello*, 2009; *Sims et al.*, 2010). One such family is the Interleukin-1 family (IL-1) of cytokines, where 11 members were already identified: IL-1α, IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-18, IL-33 and IL-1(F5-F10, reviewed in *Dinarello*, 2009; *Sims et al.*, 2010). The different members of this family have been identified to possess either pro- and anti-inflammatory properties, as shown in Table 1. As an example, IL-1Ra can bind to the IL-1 receptor (IL-1R) at higher affinity than the IL-1 itself, therefore reducing IL-1α and IL-1β responses (*Arend et al.*, 1985; reviewed in *Dinarello*, 2009). In opposition, IL-1β has been extensively studied as an important pro-inflammatory mediator released by

monocytes and tissue macrophages following an Inflammatory stimulus, such as a bacterial challenge (reviewed in *Dinarello*, 2009; *Sims et al.*, 2010).

Table 1 – IL-1 family members and their roles in Inflammation. (Adapted from *Dinarello*, 2009).

Name	New Name (Sims et al., 2001)	Property
IL-1α	IL-1F1	Agonist
IL-1β	IL-1F2	Agonist
IL-1Ra	IL-1F3	Receptor antagonist
IL-18	IL-1F4	Agonist
FIL1δ	IL-1F5	Anti-Inflammatory
FIL-1ε	IL-1F6	Agonist
IL-1H4, IL-1ζ	IL-1F7	Anti-Inflammatory
IL-1H2	IL-1F8	Agonist
IL-1ε	IL-1F9	Agonist
IL-1Hy2	IL-1F10	Receptor antagonist (?)
IL-33	IL-1F11	Agonist

Interleukin-1, as the name suggests, was the first interleukin identified, the so-called fever-producing endogenous pyrogen, and represents one of the most important mediators of Inflammation and host responses to infections (reviewed in *Dinarello*, 2004; *Sims et al.*, 2010). The biological activity of IL-1 is mediated by two cytokines, IL-1α and IL-1β, produced from two different genes both located at chromosome 2. The aminoacid homology between IL-1α and IL-1β is 22% (reviewed in *Burger et al.*, 2006). These two cytokines bind to the same receptor complex, the IL-1 receptor type-I (IL-1RI), thus having identical biological activities (*Sims et al.*, 1988; reviewed in *Dinarello*, 2009; *Sims et al.*, 2010). Although, besides their similarities, they differ in several ways such as their

expression profile. Whereas IL-1α expression is widespread (e.g. keratinocytes and endothelial cells), IL-1β expression is more confined to Immune system cells, such as monocytes and macrophages (*Dinarello*, 1996; *Dinarello*, 2009). Interleukin-1α is generally associated with the plasma membrane of the producing cells, therefore acting mainly at a local level (*Kurt-Jones et al.*, 1985; reviewed *Dinarello*, 2009). In opposition, IL-1β is secreted and circulates systemically, therefore stimulates both local and systemic responses, and represents one of the most important mediators of Inflammation and host responses to infections (*Kurt-Jones et al.*, 1985; reviewed in *Dinarello*, 2004; *Dinarello*, 2009). IL-1β promotes the recruitment of Inflammatory cells at the site of Inflammation due to its ability to increase the expression of adhesion molecules on endothelial cells and through the release of chemokines by stromal cells, which will promote the infiltration of Inflammatory and immunocompetent cells from the circulation into the site of infection (reviewed in *Gabay et al., 2010; Sims et al., 2010*). Interleukin-1β was also shown to be involved in the activation of the expression of other pro-inflammatory mediators, such as the Tumour Necrosis Factor (TNF) and IL-6 (reviewed in *Gabay et al., 2010*). Several other biological effects have also been attributed to IL-1β, however, besides its beneficial role in mediating host responses to microbial invasion, IL-1β can also have detrimental effects, such as in chronic Inflammatory diseases and septic shock, discussed further (reviewed in *Church et al.*, 2008; *Martinon et al.*, 2009).

Interleukin-1β is expressed at low levels under normal conditions and requires induction at both the transcription and translational levels (reviewed in *Dinarello*, 2009). Augmented IL-1β production has been found in patients with viral, bacterial, fungal and parasitic infections, as a result of Innate Immune response activation via recognition of different PAMPs by several PRRs (reviewed in *Dinarello*, 2009; *Martinon et al.*, 2009). Following a bacterial challenge, several

PRRs and signaling pathways become activated, that will increase the production and release of this cytokine. Interleukin-1β is synthesized as a 31 kDa precursor (pro-IL-1β) that needs to be further cleaved in order to generate its mature and secreted 17 kDa form (Figure 1, *Mosley et al.*, 1987; *Black et al.*, 1988; *Jobling et al.*, 1988; reviewed in *Martinon et al.*, 2009). Thus, IL-1β secretion is normally described as a "two-step" mechanism, requiring production and a final processing step, prior to its release (reviewed in *Eder*, 2009).

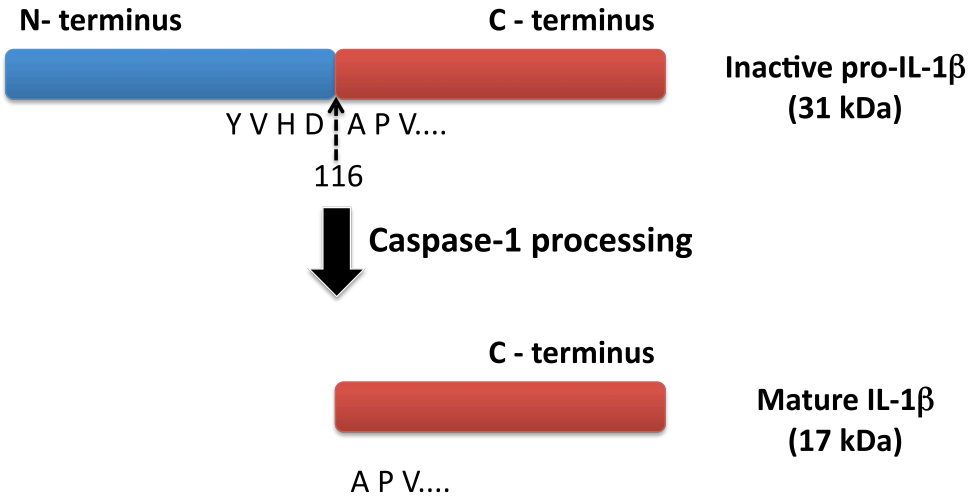


Figure 1 – IL-1β processing. The inactive pro-IL-1β (31 kDa) is cleaved at the residue 116 by Caspase-1, originating the mature and secreted form (17kDa). Adapted from (*Dinarello*, 2009).

The processing step required for the activation and release of IL-1β is mostly mediated by the IL-1-converting enzyme, Caspase-1 (Figure 1, *Cerretti et al.*, 1992; *Thornberry et al.*, 1992; *Wilson et al.*, 1994; reviewed in *Dinarello*, 2009). Though, in resting cells, Caspase-1 is produced as a catalytically inactive pro-form that also requires an activation step in order to be able to process pro-IL-Iβ into the active IL-1β. Activation of Caspase-1 is regulated by a multimeric

cytosolic protein complex, called the Inflammasome (*Martinon et al.*, 2002; reviewed in *Martinon et al.*, 2009). The formation of the Inflammasome triggers an autocatalytical activation of Caspase-1, mediated by the oligomerization of two or more Caspase-1 monomers, resulting in the cleavage of the pro-enzyme into a 20kDa (p20) and a 10-kDa (p10) subunits. Caspase-1 becomes active upon the assembling of two heterodimers of p20 and p10 subunits, containing two active sites (*Thornberry et al.*, 1992; *Wilson et al.*, 1994; reviewed in *Stutz et al.*, 2009). Caspase-1 can also cleave other members of the IL-1 family of cytokines, such as IL-18 and IL-33 (reviewed in *Martinon et al.*, 2009; *Netea et al.*, 2010). The mechanisms that lead to Inflammasome and Caspase-1 activation will be discussed further in detail.

The mechanism leading to the secretion of mature IL-1β, and other pro-Inflammatory cytokines, is also not yet well understood. Despite the identification of several possible mechanisms, such as by the usage of unidentified transporters at the plasma membrane, secretion of multivesicular bodies containing exosomes or as a passive mechanism as a result of cell lysis (*MacKenzie et al.*, 2001; *Andrei et al.*, 2004; *Brough et al.*, 2007; *Qu et al.*, 2007; reviewed in *Eder*, 2009), no unifying picture is yet drawn.

The IL-1β activation process has been extensively studied, and alternative mechanisms which do not involve Inflammasome activation or Caspase-1 were also described (reviewed in *Netea et al.*, 2010). Several studies have identified Proteinase-3, a neutrophil and macrophage derived serine protease, Elastase and Cathepsin-G as IL-1β processing enzymes (*Coeshott et al.*, 1999; *Greten et al.*, 2007; reviewed in *Dinarello*, 1996; *Netea et al.*, 2010). Nonetheless, these alternative mechanisms are important players in the regulation of IL-1β secretion, they are not under the scope of my thesis, which will focus only on Inflammasome dependent IL-1β processing.

1.1.1 - The "Inflammable" players

1.1.1.1 - The cells

During the course of the Inflammation process, several cell types are important at the different stages of this Immune response. Among the different cell types involved in an Inflammatory response are the phagocytic leukocytes (e.g. monocytes, macrophages and neutrophils), professional antigen presenting cells (e.g. Dendritic Cells, DCs) and cells from the skin and the epithelia that line our internal organs such as gut and lungs. The epithelia surfaces of the body are the first lines of defense against infection providing a physical barrier between the internal milieu and the external environment. Besides providing this physical barrier, epithelial cells also produce chemical substances that are microbicidal or inhibit microbial growth, as the case of the antibacterial enzymes (lysozyme and phospholipase A that are secreted in tears and saliva), and the antimicrobial peptides (reviewed in *Schroder et al.*, 2006; *Nevalainen et al.*, 2008). However, during Inflammation, several other cell types play important roles, as the case of the myeloid cells. These myeloid cells include mononuclear phagocytes and polymorphonuclear phagocytes. The polymorphonuclear phagocytes include the neutrophils, basophils and eosinophils (reviewed in *Dale et al.*, 2008; *Blanchard et al.*, 2009; *Sullivan et al.*, 2009). The mononuclear phagocytes are the macrophages, which are derived from the monocytes, and the closely related dendritic cells, also from myeloid lineage. During acute Inflammation, is often observed a rapid influx of blood granulocytes, typically neutrophils, followed by monocytes that mature into Inflammatory macrophages that subsequently proliferate and thereby affect the functions of resident tissue macrophages (reviewed in *Serhan et al*., 2005). The

resolution of Inflammation can occur if granulocytes are eliminated and the tissue mononuclear cell population (macrophages and lymphocytes) returns to a normal pre-inflammation state number and phenotype (reviewed in *Serhan et al., 2005; Medzhitov, 2008*).

Despite the fact that several Immune cell types play an important role in this process, in this thesis, I will focus mainly the monocytes and macrophages, which are the main producers of the pro-inflammatory cytokine IL-1β (reviewed in *Dinarello*, 2009; *Eder*, 2009).

Monocytes are usually defined as a pleomorphic and pleiotropic population of circulating mononuclear cells, which contribute to antimicrobial defense by supplying tissues with macrophage and DC precursors (*van Furth et al.*, 1968; *van Furth et al.*, 1972; *Gordon et al.*, 2005).

"The function and fate of the monocytes in the circulation is not clearly understood. In vitro these cells can mature into a cell with characteristics of tissue macrophages and similar transformations were shown to occur in vivo at extravascular sites. Recently it has been reported that macrophages in Inflammatory exudates originate from monocytes."

Ralph van Furth, 1968

The monocytes originate in the bone marrow from a common myeloid progenitor that is shared with neutrophils (*Volkman et al.*, 1965), and are released into the peripheral blood, where they circulate for several days before entering tissues and replenish the tissue macrophage populations (reviewed in *Taylor et al.*, 2003; *Serbina et al.*, 2008). The morphology of mature monocytes in the peripheral circulation is heterogeneous, and these cells constitute approximately 5-10% of peripheral-blood leukocytes in humans (*Gordon et al.*, 2005). Monocytes do not proliferate in steady state (reviewed in *Auffray et al.*, 2009). These cells are

equipped with chemokine receptors and adhesion receptors that allow the migration from blood into tissues during infection (reviewed in *Serbina et al.*, 2008; *Geissmann et al.*, 2010). As an example, after bacterial infection, such as *Listeria monocytogenes*, there is an influx of monocytes to the site of infection, occurring normally 72 to 96 h following infection (reviewed in *Serbina et al.*, 2008) where, according to the Inflammatory milieu, they differentiate into Inflammatory DCs and macrophages (reviewed in *Serbina et al.*, 2008; *Geissmann et al.*, 2010). Besides differentiation into macrophages, monocytes can also exert bactericidal functions by producing several cytokines (e.g. IL-1β, IL-8) or toxic molecules, such as reactive nitrogen intermediates (RNIs) or reactive oxygen intermediates (ROIs, *Serbina et al.*, 2008).

Macrophages are commonly defined as the "phagocytic cells *per excellence*". These phagocytic cells play a key role in Innate immunity due to their capability to recognize, ingest and destroy many pathogens without the aid of an adaptative Immune response. The important role of macrophages in phagocytosis is in the origin of its name, the term macrophages (from the Greek for "large eaters", reviewed in *Pollard*, 2009). Phagocytosis has been largely studied, however *Metchnikov*, for the majority considered the "father" of Innate immunity, has already described its importance in the beginning of the XX century.

"Whenever the organism enjoys immunity, the introduction of infectious microbes is followed by the accumulation of mobile cells, of white corpuscles of the blood in particular which absorb the microbes and destroy them. The white corpuscles and the other cells capable of doing this have been designated "phagocytes", i.e. devouring cells, and the whole function that ensures immunity has been given the name of "phagocytosis"."

Metchnikoff, *Nobel Lecture in 1908*

Macrophages mature continuously from monocytes that leave the circulation to migrate into tissues throughout the body (reviewed in *Pollard*, 2009). In mammals, macrophages are found in all tissues. In some tissues they constitute 10-20% of all cells, as for example microglial cells in the brain and Kupffer cells in the liver, whereas in other tissues, such as musculo-skeletal junctions, they are rare (*Perry et al.*, 1985; *Sasmono et al.*, 2003; *Pollard*, 2009). Macrophages, as monocytes, upon bacterial recognition are able to produce several adhesion molecules, cytokines, chemokines and other signaling molecules (reviewed in *Gordon et al.*, 2005).

1.1.1.2 - The Sensors – PRRs

Over the last years, a large number of studies in Immunology and Microbiology have unveiled pivotal roles of the host Innate Immune system in sensing microbial infections via specific Innate Immune receptors, the Pattern Recognition Receptors (PRRs, *Janeway*, 1989). The PRRs act as a molecular switch to trigger Innate Immune activation and tightly regulate the subsequent adaptative Immune responses to microbial infections. These receptors can be expressed at the cell surface, in intracellular compartments, or secreted into the bloodstream and tissue fluids (*Medzhitov et al.*, 1997a; *Janeway et al.*, 2002). The PRRs are germline encoded, and each receptor has broad specificities for conserved and invariant features of microorganisms, sometimes referred as pathogen-associated molecular patterns (PAMPs), although being present in

pathogenic and non-pathogenic microorganisms (*Janeway et al.*, 2002). These molecules are vital for the survival of the microorganism, and as such are unlikely to vary in their structures because that could be disadvantageous for the microorganisms. Another interesting aspect of the PAMPs is the fact of being invariant among microorganisms of a given class and products of pathways unique to microorganisms, allowing in this way the discrimination between the self and non-self molecules (*Medzhitov et al.*, 1997a; *Medzhitov*, 2007). Examples of PAMPs include Lipopolysaccharide (LPS), bacterial lipoprotein, peptidoglycan (PGN), among others. However, PAMPs are not the only triggers of the Innate immunity, as suggested by the Danger Model of Immune System activation (*Matzinger*, 2001; *Matzinger*, 2002b). This model suggests that the Immune system is more concerned with damage than with the recognition of "foreignness", and is called into action by alarm signals from the injured tissues, rather than the recognition of non-self molecules. Several "self" molecules have now been shown to be released by injured tissues and to activate an Innate Immune response. These are called Damage Associated Molecular Patterns (DAMPs), such as DNA, RNA or uric acid, which should not normally be present outside the cells or at certain locations within the cell under physiological conditions (*Matzinger*, 2002a; *Shi et al.*, 2003; *Bianchi*, 2007).

Several families of PRR have so far been identified differing in their cellular and subcellular location, and the signaling pathways elicited, which is reflected in the different biological roles (reviewed in *Janeway et al.*, 2002; *O'Neill*, 2008; *Werts et al.*, 2006). In fact, the subcellular location of the receptors has been used to distinguish between the several families of PRRs, the membrane bound and the cytosolic families of receptors. The transmembrane Toll-like Receptors (TLRs) and the C-type Lectin Receptors are located at the plasma membrane and on internal membranes, such as the endosome. Whereas, the members of cytosolic receptors comprise the Retinoic Acid-inducible Gene-I like

Helicases (RLRs) and the Nucleotide-Binding Domain Leucine-rich Repeat-containing Receptors (NLRs). Despite the importance of all the PRRs in the generation of an Immune response, this thesis will focus mainly on the TLR family of membranar receptors and the cytosolic family or PRR, the NLRs.

1.1.1.2.1 - Toll-like Receptors (TLRs)

The Toll discovery was achieved in *Drosophila* (*Hashimoto et al.*, 1988), as one of the 12 maternal effect genes that function in a pathway required for dorso-ventral axis formation in fly embryos (reviewed in *Belvin et al.*, 1996). Homologues of *Drosophila* Toll have been identified in mammals and are referred to as Toll-like Receptors (TLRs) (reviewed in *Janeway et al.*, 2002; *O'Neill*, 2008). TLRs are transmembrane proteins characterized by an ectodomain that contains varying numbers of leucine-rich-repeat (LRR) motifs and a cytoplasmic signaling domain named Toll/IL-1R homology (TIR) domain (*Akira et al.*, 2004; *Creagh et al.*, 2006). The first human Toll to be characterized was the TLR4, which was shown to induce, like its *Drosophila* homologue, the activation of the nuclear factor-kB (NF-kB) signaling pathway (*Medzhitov et al.*, 1997b). Since then, several members of this family have been described (reviewed in *Ishii et al.*, 2008; *O'Neill*, 2008). To date 10 members of TLR family have been found in human, and a series of genetic studies have identified their ligands (Figure 2, reviewed in (*Akira et al.*, 2004)). For example, LPS of gram-positive bacteria is recognized by the TLR4 (*Poltorak et al.*, 1998; *Hoshino et al.*, 1999). TLR2, in concert with TLR1 or TLR6, recognizes various bacterial components, including peptidoglycan, lipopeptide and lipoprotein of Gram-positive bacteria and *Mycoplasma* lipopeptide

(*Aliprantis et al.*, 1999; *Ozinsky et al.*, 2000; *Takeuchi et al.*, 2000; *Morr et al.*, 2002). Moreover, TLR1/TLR2 and TLR2/TLR6 dimers can discriminate between triacyl lipopeptide (*Takeuchi et al.*, 2002) and diacyl lipopeptide (*Takeuchi et al.*, 2001), respectively. TLR3 is involved in the recognition of viral double stranded RNA (*Alexopoulou et al.*, 2001). TLR5 recognizes bacterial flagellin (*Hayashi et al.*, 2001). Different ligands have been found to be recognized by TLR7 and TLR8, among them are viral single-stranded RNA (*Diebold et al.*, 2004; *Heil et al.*, 2004) and several synthetic compounds such as Imidazoquinoline, Loxoribine and bropirimine (*Hemmi et al.*, 2002; *Jurk et al.*, 2002; *Heil et al.*, 2003). TLR9 recognizes bacterial and viral CpG DNA motifs (*Hemmi et al.*, 2000; *Lund et al.*, 2003) and also malaria pigment hemozoin (*Coban et al.*, 2005). TLR11 was shown to recognize Uropathogenic bacteria (*Zhang et al.*, 2004). Upon recognition of the microbial pathogens, TLRs trigger intracellular signaling pathways that result in the induction of Inflammatory cytokines, type I interferon (IFN), chemokines and co-stimulatory molecules (reviewed in *Akira et al.*, 2004). The signaling pathways elicited by the recognition of different PAMPs by the different TLRs will be discussed further.

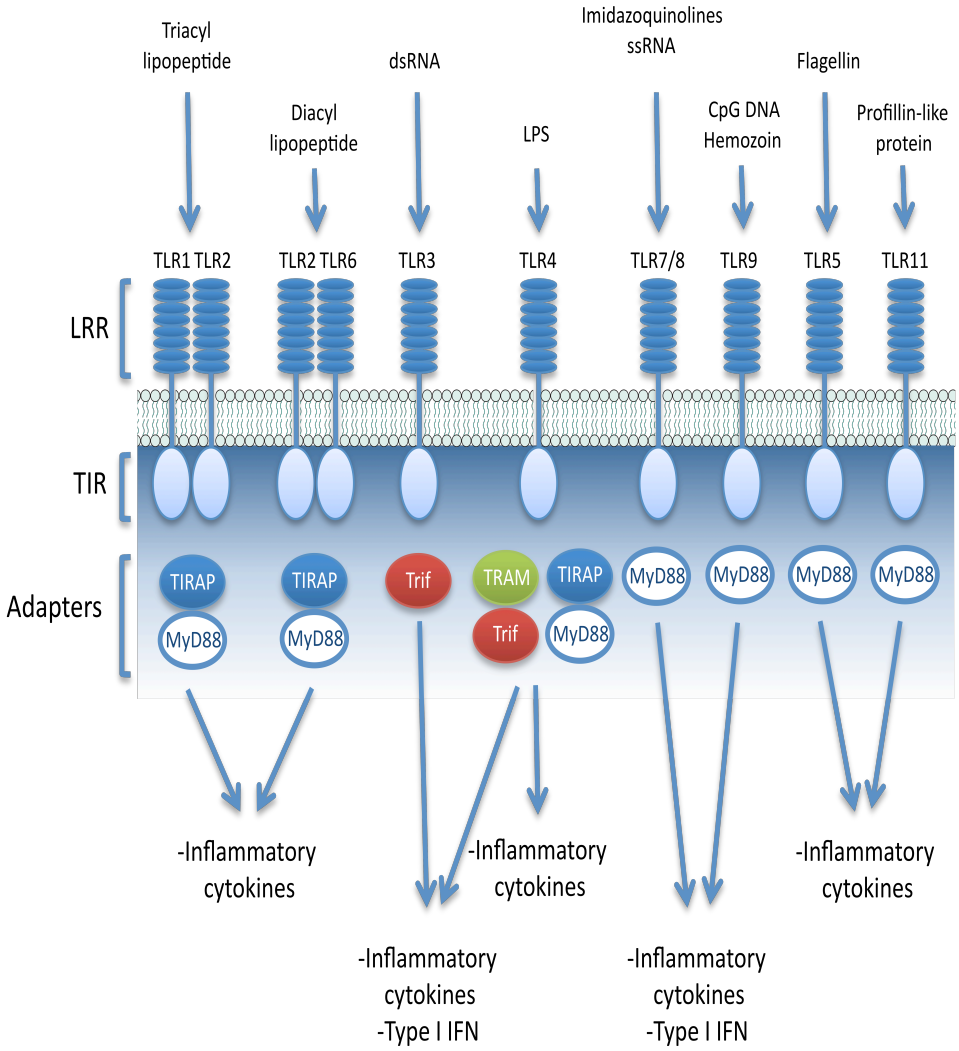


Figure 2 – TLR mediated Immune responses. The different TLRs recognize different molecules (PAMPS and DAMPS), such as bacterial LPS and flagellin. The dimerization and the recruitment of different adaptor molecules allow the induction of different signaling pathways. Some receptors use the MyD88 and TIRAP/MAL adapters, whereas some use Trif. Upon activation of a MyD88-dependent pathway, an Inflammatory response is elicited, while Trif mainly mediates type I IFN responses. Exceptions can occur, for example, TLR7/8 and TLR9 induce type I IFN in a MyD88-dependent manner in plasmacytoid Dendritic cells. Adapted from (*Kawai et al.*, 2006).

1.1.1.2.1.1 - TLR signaling pathways

TLRs initiate shared and distinct signaling pathways by recruiting different combinations of four signaling adaptor molecules (Figure 2), the TIR domain-containing adaptor molecules: MyD88, TIRAP, TRIF and TRAM (*Akira et al.*, 2004). MyD88 is the most common adapter, which is used by all TLRs, with the exception of TLR3. The signaling pathway activated by TLR2 and TLR4 also use TIRAP, while TRAM is only used by TLR4. TRIF is used by TLR3 and TLR4 (*Kawai et al.*, 2006). These signaling pathways activate the NF-κB and activator protein-1 (AP-1) transcription factors, that are common to all TLRs, leading to the production of Inflammatory cytokines (such as Interleukin 1β, IL-1β) and several chemokines (reviewed in *Akira et al.*, 2004). TLRs 3, 4, 7, 8, and 9 also activate interferon regulatory factor 3 (IRF3) and/or IRF7, leading to the production of type I IFNs such as IFNβ and IFNα (*Kawai et al.*, 2008). Subsequently, the cytokines and chemokines initiate and amplify Inflammatory responses by recruiting and activating various Innate Immune cells such as monocytes, neutrophils, and natural killer cells (*Iwasaki et al.*, 2004). Type I IFNs can induce an antiviral state in most cells and, in addition, have diverse functions in the development of adaptive immunity (*Stark et al.*, 1998; *Iwasaki et al.*, 2004; *Theofilopoulos et al.*, 2005; *Pulendran et al.*, 2006).

As mentioned above, TLRs can trigger both MyD88 dependent and MyD88 independent signaling cascades (reviewed in *Kawai et al.*, 2006). Upon TLR stimulation, in a MyD88 dependent pathway, TIRAP and MyD88 are recruited via homophilic Toll/interleukin-1 receptor (TIR) domain interactions, allowing the association of IRAK4 (IL-1R-associated-kinase 4) with the receptor complex (*Muzio et al.*, 1997; *Wesche et al.*, 1997; *Burns et al.*, 1998). IRAK4 then phosphorylates IRAK1 (*Li et al.*, 2002). Tumour-necrosis-factor-receptor-

associated-factor 6 (TRAF6) is also recruited to the receptor complex, by associating with phosphorylated IRAK1 (*Ye et al.*, 2002). Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-β-activated-kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1 (*Shibuya et al.*, 1996; *Takaesu et al.*, 2000). IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating-enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1 (*Chen*, 2005). TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-kB (IkB)-kinase complex), which consists of IKK-α, IKK-β and IKK-γ (also known as IKK1, IKK2 and nuclear factor-kB (NF-kB) essential modulator, NEMO, respectively (*Chen*, 2005; *Kawai et al.*, 2006)). The IKK complex then phosphorylates IkB, which leads to its ubiquitination and subsequent degradation (reviewed in *Mankan et al.*, 2009; *Skaug et al.*, 2009). This allows NF-kB to translocate to the nucleus and induce the expression of its target genes, among them is the pro-inflammatory cytokine IL-1β (*Dinarello*, 2009; *Mankan et al.*, 2009). TAK1 also activates MAP kinases such as c-Jun N-terminal kinases (JNKs), p38 and probably extracellular signal-related kinases (ERKs), leading to the activation of AP-1 and thus the regulation of Inflammatory cytokine expression (*Wang et al.*, 2001; *Sato et al.*, 2005). The transcription factor IRF5 is also critical for the expression of Inflammatory cytokines downstream of the TLR-MyD88 signaling pathway, initiated by TLRs 4,5,7 and 9 (*Sato et al.*, 2005). Thus, this classical MyD88 dependent pathway induces the expression of various Inflammatory cytokines (e.g.IL-1β) by a combination of NF-kB, AP-1 and IRF-5 (Figure 3).

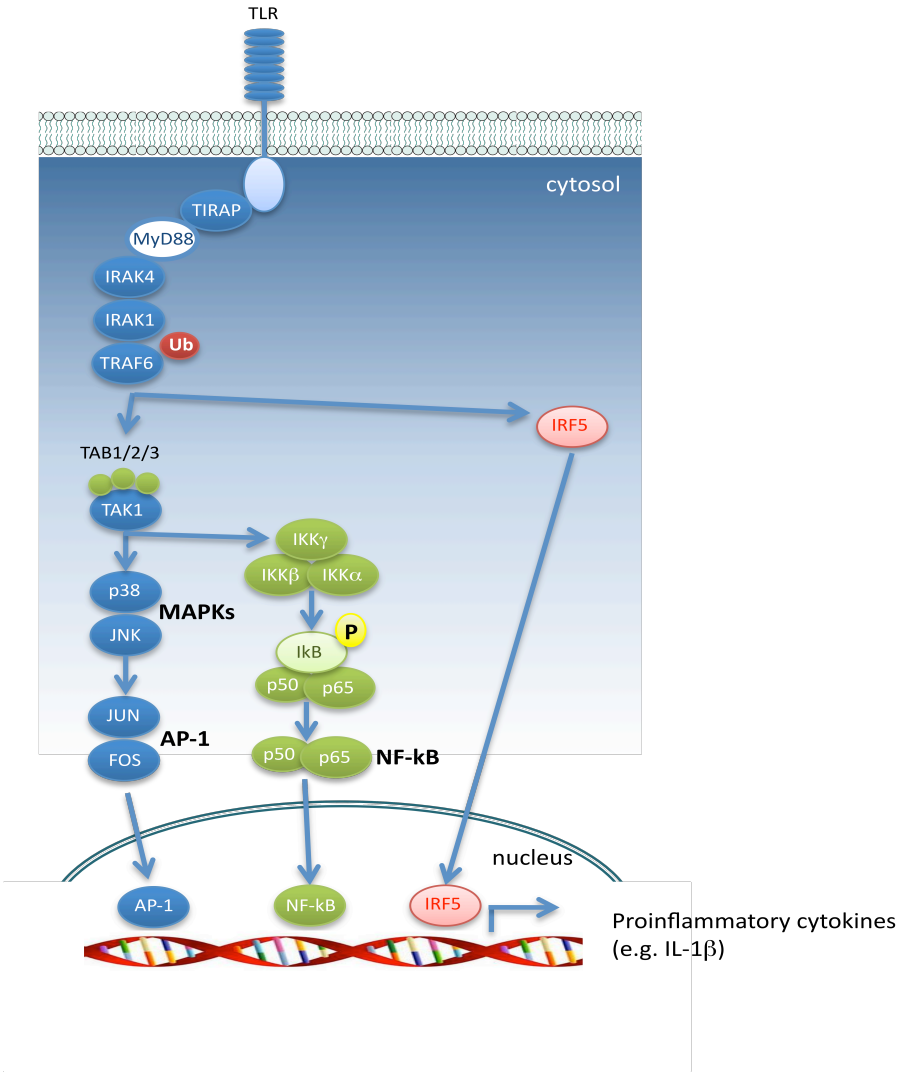


Figure 3 - TLR MyD88 dependent signaling pathways. Upon TLR stimulation MyD88 and TIRAP are recruited via homophilic TIR-TIR interactions. MyD88 then associates with IRAK4 and IRAK1, leading to sequential activation of IRAK4, IRAK1 and TRAF6. Ubiquitinated TRAF6 can be recruited to the TAK1-TAB1/2/3 complex by binding to TAB2, promoting TAK1 activation and therefore MAP kinases and NF-kB pathways, leading to the expression of Inflammatory cytokines. TAK1 also activates MAP kinases such as JNK, p38 and ERK, leading to the activation of AP-1, regulating Inflammatory cytokine expression. The transcription factor IRF5 can also be activated in a MyD88 dependent signaling pathway, although the detailed mechanism is not yet known. Adapted from (*Lee et al.*, 2007).

1.1.1.2.1.2 - Regulation

Several regulators have been identified to function dampening responses of TLRs (reviewed in *Liew et al.*, 2005). Several Decoy receptors have been found (e.g.TLR2 and TLR4) to be produced naturally during severe infection, and to block the interactions between the bacterial ligands and their respective TLRs (*Iwami et al.*, 2000; *LeBouder et al.*, 2003). Certain negative regulators of TLR signaling are upregulated following TLR stimulation, for example, ST2 (which sequester MyD88 or TIRAP, *Kumar et al.*, 1997; *Saccani et al.*, 1998; *Brint et al.*, 2004), MyD88s (the short isoform of MyD88 that abrogates NF-kB activation that is activated by the TLRs that signal via MyD88, *Janssens et al.*, 2002; *Burns et al.*, 2003; *Janssens*, 2003), IRAK-M (which inhibits IRAK1 phosphorylation, *Kobayashi et al.*, 2002), suppressor of cytokine signaling-1 (SOCS1, which mediates TIRAP degradation, *Mansell et al.*, 2006) and A20 (which de-ubiquitinates TRAF6, *Boone et al.*, 2004). These negative regulators attenuate the TLR response via a negative feedback loop; although, others exist that target TLR signaling by downregulating the transcription or translation of TLR genes or by the degradation of TLR proteins. Among these are TRIAD3A (which ubiquitinates TLRs, targeting them for proteasomal degradation, *Chuang et al.*, 2004), Toll-interacting protein (TOLLIP, which controls the magnitude of responses to IL-1 and LPS, *Burns et al.*, 2000; *Bulut et al.*, 2001; *Zhang et al.*, 2002) and IRF4 (which competes with IRF5 for MyD88 binding, *Negishi et al.*, 2005), among others. A more detailed and complete list of negative regulators of TLR signaling can be seen in Figure 4 (reviewed in *Lee et al.*, 2007).

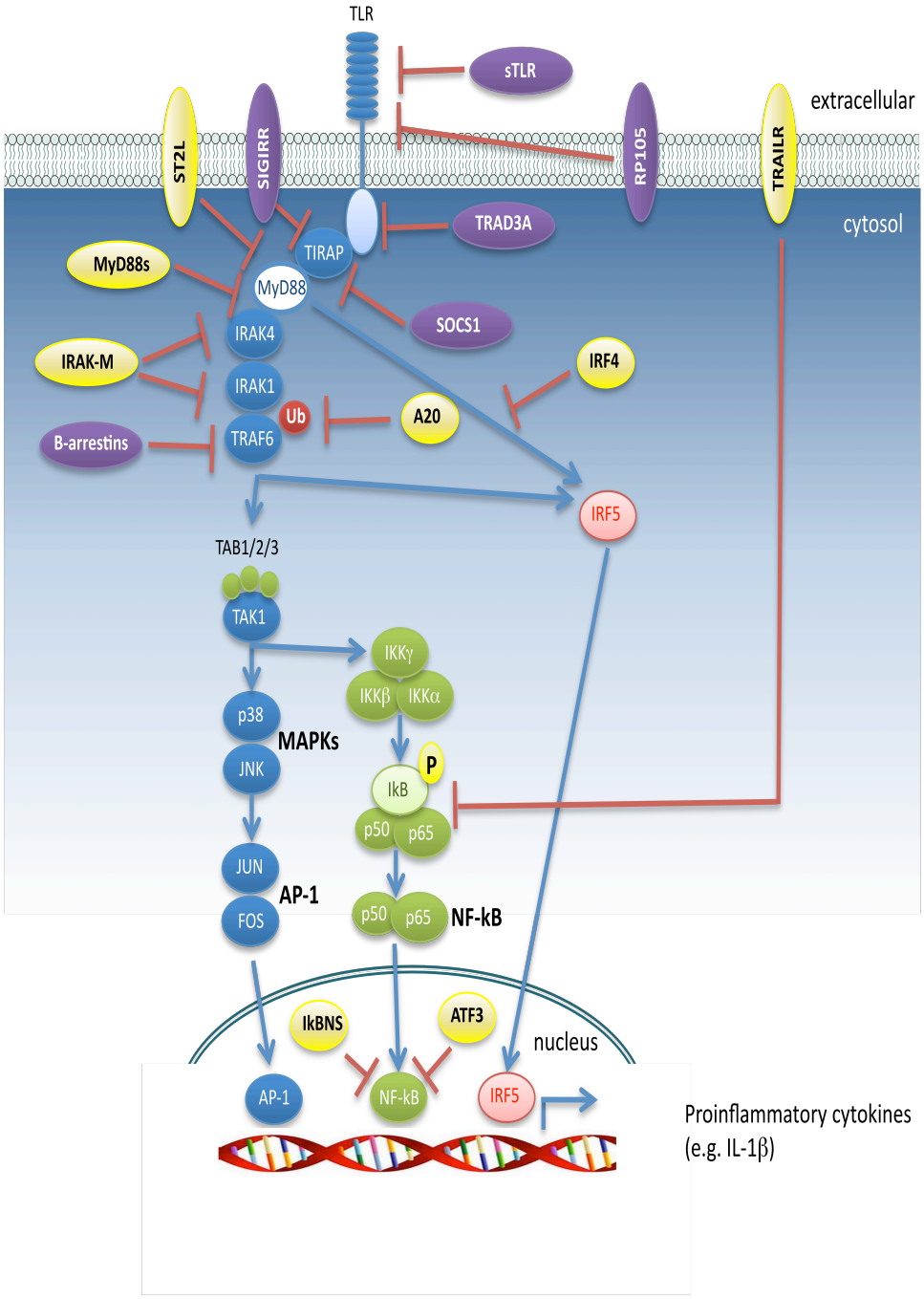

TLR
sTLR
extracellular
ST2L
SIGIRR
RP105
TRAILR
cytosol
TRAD3A
TIRAP
MyD88s
MyD88
SOCS1
IRAK4
IRAK-M
IRF4
IRAK1
Ub
A20
TRAF6
B-arrestins
IRF5
TAB1/2/3
TAK1
IKKγ
IKKβ
IKKα
p38
MAPKs
JNK
P
IkB
p50
p65
JUN
AP-1
FOS
p50
p65
NF-kB
IkBNS
ATF3
nucleus
AP-1
NF-kB
IRF5
Proinflammatory cytokines
(e.g. IL-1β)

Figure 4 - Negative regulation of TLR MyD88 dependent pathway. The negative regulator proteins that act at early time points and/or interfere with primary TLR signaling are represented in violet, and those acting in a negative feedback mechanism and/or at late time points are depicted in yellow. Soluble TLR (sTLR) may act as a decoy receptor, interfering with signal generation via the specific TLR. The mechanisms of TLR signaling dampening differ among the different negative regulator proteins: Toll/IL-1R homology (TIR) domain-containing transmembrane proteins STL2L (sequesters MyD88 and TIRAP) and SIGIRR (TLR signalosome assembly interference, by interacting with TLR4, IRAK and TRAF6); TRAILR, a tumor necrosis factor (TNF) superfamily member (stabilizes IkBα at later times after TLR stimulation, interfering with NF-kB activation); RP105, a TLR homolog lacking a signaling domain (interferes with the formation of the LPS-signaling receptor complex TLR4-MD2); MyD88s, a splice variant of MyD88 (prevents the recruitment of IRAK4); IRAKM, an IRAK1 homolog (inhibits the dissociation of IRAK1 and IRAK4 from the TLR signaling complex); the ubiquitin-modulating enzymes A20 (cleave the ubiquitin chain of TRAF6, dampening further signaling activation); TRIAD3A (bind to the cytoplasmic domain of TLR and stimulate its ubiquitin-dependent degradation); SOCS1, suppressor of cytokine signaling-1 (mediates TIRAP degradation); IRF4, interferon regulatory factor-4 (competes with IRF5 for MyD88 binding); the activating transcription factor-3, ATF3 (suppresses the expression of certain TLR induced genes, probably due to alteration of the chromatin structure, preventing the access of NF-kB to the promoters); IkBNS (inhibits NF-kB activity in the nucleus, possibly due to interaction with p50 protein) and β-arrestins (interact with TRAF6, inhibiting its ubiquitination and this NF-kB and AP-1 activation). Adapted from (*Lee et al.*, 2007).

1.1.1.2.2 - NOD-like Receptors (NLRs)

Several studies have demonstrated the importance of the homologous and heterologous cooperation of TLRs and other PRRs in the detection and triggering of Innate Immune responses to microbial infections (reviewed in *Creagh et al.*, 2006; *Trinchieri et al.*, 2007). Among those PRRs are the members of the NOD-like receptor family (NLRs, reviewed in *Inohara et al.*, 2005; *Martinon et al.*, 2005). NOD-like receptors are normally described as a specialized group of intracellular proteins that play a critical role in the regulation of the host Innate Immune response. They act as scaffolding proteins that assemble different signaling platforms triggering NF-kB and mitogen-activated protein kinase (MAPK) signaling pathways and control the activation of Inflammatory Caspases (*Inohara et al.*, 2005).

The NLR family is characterized by a tripartite domain structure (Figure 5), which shares structural similarity with a subclass of plant disease resistance (R) genes (*Hammond-Kosack et al.*, 1997; *Janeway et al.*, 2002). The N-terminus is composed of an effector domain, normally a Pyrin domain (PYD) or Caspase-recruitment domain (CARD), which is involved in homotypic and heterotypic binding. In this N-terminal domain other domains have been reported, such as the Baculovirus IAP Repeat (BIR). A nucleotide-oligomerization domain (NOD/NACHT) follows the effector domain, which might function in the regulation of self-oligomerization. The C-terminus is mainly composed of a series of LRR, similar to the TLR LRR motifs (*Bella et al.*, 2008), although some exceptions were reported to occur, such as the HIN200 domain present in the AIM2 (*Ludlow et al.*, 2005). The LRR domain has been implicated in ligand sensing and autoregulation of NLRs, although the precise mechanism of how NLR LRRs sense their ligands is largely unknown.

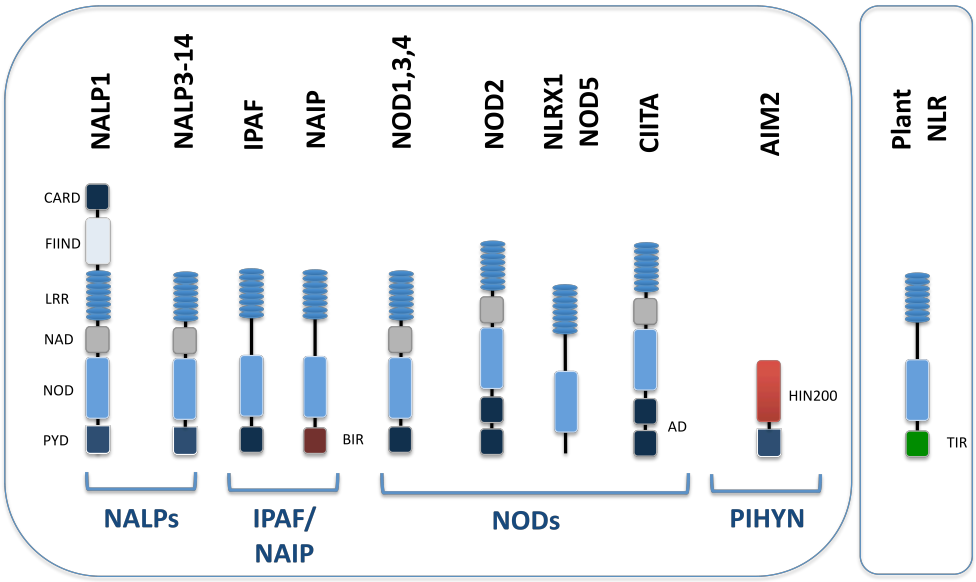


Figure 5 – Domain structures of the different NLRs. NLRs are characterized by a tripartite structure: the ligand-sensing leucine rich repeats (LRRs); the NOD domain, which is responsible for the oligomerization capacity of the NLRs; and the N-terminal effector domain, that can be a pyrin domain (PYD), CARD or BIR domain. NLRs are divided into 4 subfamilies, NALPs, IPAF/NAIP,NODs and PIHYN, which differ mainly in their N-terminal domains. (Abbreviations: CARD, Caspase recruitment domain; PYD, pyrin domain; FIIND, function to find; NOD, nucleotide-oligomerization domain; NAD, NACHT-associated domain; BIR, baculovirus IAP repeat; AD, activation domain; CIITA, MHC class II transcription activator). Adapted from (*Martinon et al.*, 2009; *Stutz et al.*, 2009).

There are more than 24 NLR proteins already described in the human genome, although little is known about their functions. The NLRs are subdivided in 4 major subfamilies (NALPs, NODs, IPAF/NAIP and PIHYN, *Tschopp et al.*, 2003; *Stutz et al.*, 2009), mainly due to their different N-terminal effector domains (Figure 5), which mediate signal transduction to downstream targets, leading to activation of Inflammatory Caspases (*Poyet et al.*, 2001; *Martinon et al.*, 2002; *Martinon et al.*, 2009) or NF-kB (NODs, *Bertin et al.*, 1999; *Inohara et al.*, 1999). NALPs represent the largest NLR subfamily and have 14 genes identified in humans (*Tschopp et al.*, 2003; *Ting et al.*, 2008). NALP proteins harbor a NOD

and LRR and are characterized by an N-terminal PYD domain. Notably, the LRR region within NALPs is organized in the genome in a very conserved and precise manner. NALP LRR regions are formed by tandem repeats of exons of exactly 171 nucleotides and are defined completely by a preserved intron/exon structure. Each exon encodes one central LRR and two halves of the neighboring LRRs (*Martinon et al.*, 2007b). This modular organization possibly allows extensive Alternative Splicing (AS) of the LRR region without destabilization of the structure and therefore maximizing variability in the ligand-sensing unit of the NALPs. A N-terminal CARD domain and three BIR domains characterize IPAF and NAIP, respectively (*Roy et al.*, 1995; *Inohara et al.*, 2005). The third NLR subfamily, NODs, includes the CARD containing NLRs such as the NOD1, NOD2, NOD3, NOD4 and CIITA. This third subclass of NLRs also contains the NLRX1 and NOD5 without a defined N-terminal domain (*Martinon et al.*, 2005; *Proell et al.*, 2008). Recently, another member of this family of PRR was discovered, called the Protein Absent in melanoma 2 (AIM2, *Burckstummer et al.*, 2009; *Fernandes-Alnemri et al.*, 2009; *Hornung et al.*, 2009; *Roberts et al.*, 2009) which is the only member of the PIHYN subclass so far described. The PIHYN subclass is characterized by the presence of an HIN200 domain (*Roberts et al.*, 2009) at the C-terminus, whereas all the other NLRs described so far present a LRR domain. Several are the proteins described to possess an HIN200 (PIHYN family, reviewed in *Ludlow et al.*, 2005), however, AIM2 is the only member described to be involved in Immune responses. In contrast to the majority of the identified PIHYN family members, AIM2 is the only showing cytoplasmic location. Moreover, the pyrin domain of AIM2 is more closely related to those of the NLRs than those of the other PYHIN family members (*Hornung et al.*, 2009; *Hornung et al.*, 2010).

The different NLRs differ not only in their structure but also in the PAMPS they recognize, as well as in the signaling cascades elicited upon activation. NOD1 and NOD2 have been shown to recognize peptidoglycan (PGN)-

derived products (reviewed in *Inohara et al.*, 2005). NOD2 is a general sensor for both Gram-positive and Gram-negative bacteria since biochemical and functional analyses have identified muramyl dipeptide (MDP), the minimal motif common to all peptidoglycans, as the essential structure recognized by NOD2 (*Inohara et al.*, 2003; *Franchi et al.*, 2009). In contrast to NOD2, NOD1 presents a strict sensing specificity towards DAP-type peptidoglycan (*Chamaillard et al.*, 2003a; *Chamaillard et al.*, 2003b). Moreover, it has been shown that the human NOD1 detects a single muropeptide GM-TriDAP, which is produced as a peptidoglycan degradation product in Gram-negative bacterial metabolism (*Benko et al.*, 2008). Stimulation of NOD1 or NOD2 results in the activation of NF-kB and MAPKs, which drive the transcription of numerous genes involved in both Innate and adaptative Immune responses (*Hayden et al.*, 2004). Among the most studied NLRs are the members that have been shown to be important in the regulation and activation of pro-inflammatory Caspases, such as NALP3, NALP1 and IPAF (reviewed in *Tschopp et al.*, 2003). IPAF has been shown to recognize bacterial flagellin (*Franchi et al.*, 2006; *Miao et al.*, 2006). Flagellin is a bacterial protein that can self-assemble to form flagella, a macromolecular complex that plays a role in the motility of either Gram-negative or Gram-Positive bacteria. Moreover, flagellin monomers from a number of flagellated bacteria are extremely conserved, which has allowed the evolution of Innate Immune mechanism of bacterial flagellin detection (*Miao et al.*, 2007). NALP1 recognizes the Anthrax lethal toxin, from *Bacillus anthracis* (*Boyden et al.*, 2006). Anthrax lethal toxin is the major cause of anthrax-related death and so far the only toxin identified to activate NALP1. NALP3 mediates the activation of Caspase-1 in response to a variety of structurally unrelated stimuli. The repertoire of agonists capable of inducing NALP3 activation include nucleic acids such as bacterial and viral RNA (*Kanneganti et al.*, 2006a; *Kanneganti et al.*, 2006b; *Allen et al.*, 2009), monosodium urate crystals (*Martinon et al.*, 2006), changes in ion concentrations,

such as potassium (*Pétrilli et al.*, 2007), pore forming toxins such as nigericin and maitotoxin (*Mariathasan et al.*, 2006), reactive oxygen species produced following asbestos and silica exposure (*Dostert et al.*, 2008) and fungal ligands, such as zymosan and mannan (*Hise et al.*, 2009; *Lamkanfi et al.*, 2009b). The only member of the PYHIN subclass of NLRs, the AIM2, recognizes dsDNA in cytosol (*Burckstummer et al.*, 2009; *Fernandes-Alnemri et al.*, 2009b; *Hornung et al.*, 2009; *Roberts et al.*, 2009). This receptor binds directly to dsDNA, but not ssDNA through it C-terminal HIN200 domain, which results in AIM2 oligomerization and recruitment of other cytosolic proteins via homotypic PYD domain interaction, and subsequent activation of a signaling pathway (*Roberts et al.*, 2009; *Vilaysane et al.*, 2009).

The ligands that are already described to activate each NLR are showed in Table 2, however, the ligands for some NLR proteins still remain to be identified.

Table 2 - NLR family members and the PAMPS recognized. Adapted from (*Benko et al.*, 2008; *Franchi et al.*, 2009; *Mishra et al., 2010; Hornung et al., 2010*).

NLR	PAMPs recognized
NALP1	MDP, Anthrax Lethal Toxin
NALP2	?
NALP3	Bacterial RNA Viral RNA Bacterial antigens (e.g. Esat6) Uric Acid Crystals (e.g. MSU, CPPD) Bacterial cell wall components (e.g. LPS, MDP) Pore forming toxins (e.g. nigericin, maitotoxin), Fungal ligands (e.g. Zymosan and mannan) Xenogeneous particles (e.g. Asbestos, Silica)
NALP4-14	?
IPAF	Flagellin
NAIP	Flagellin
NOD1	GM-tripeptide, iE-DAP, FK156, FK565
NOD2	MDP, M-TRILys
NOD3-4	?
NLRX1	?
CIITA	?
AIM2	dsDNA

?- not yet identified

Expression patterns of most NLRs in various cell populations and tissues have not yet been studied in detail. However, the importance of NLRs in defense strategies of the body is supported by the fact that several NLRs are expressed in cell and tissues that have an important role in immunity, such as phagocytes. Some NLRs, such as NOD1, NOD2, NALP3 and NAIP are expressed in epithelial cells (*Kufer et al.*, 2005; *Kummer et al.*, 2007; *Vinzing et al.*, 2008). NALP1 is widely expressed, whereas NALP3 is expressed mainly in Immune cells, epithelial cells and osteoblasts (*Kummer et al.*, 2007; *McCall et al.*, 2008). NAIP and IPAF are expressed in the brain and in macrophages and macrophage-rich tissues such as spleen, lung, and liver (*Diez et al.*, 2000; *Poyet*, 2001). NALP5, NALP8, NALP4, NALP7, NALP10, and NALP11 are mainly expressed in germ cells and preimplantation embryos (*McDaniel et al.*, 2009). The expression profile of different NLRs has also been shown to be influenced by other PRRs, as part of a regulatory network. TLR stimulation, for example, increases the expression of NLRs, such as NOD1, NOD2, and NALP3, possibly reflecting enhancement of NLR responses after TLR stimulation (*Becker et al.*, 2007).

1.1.1.2.2.1 - NLR signaling pathways

The signaling pathways elicited upon recognition of PAMPs by the different NLR families differ, as well the outcome of that activation. As can be seen in Figure 6, ligand recognition by the NODs (NOD1 and NOD2) induces the recruitment of the CARD-containing serine/threonine kinase RICK (also called RIP2) to the receptors via prototypical CARD-CARD interaction (*Inohara et al.*, 2005; *Martinon et al.*, 2005; *Ting et al.*, 2006). Activated RICK mediates the ubiquitination of IKKγ, leading to NF-kB activation (*Abbott et al.*, 2004; *Strober et al.*, 2006). The underlying mechanism of IKKγ ubiquitination by RICK is not

known, although several potential regulators of NF-kB activity in the NOD-dependent pathways have been identified to be involved, such as TAK1, TRIP-6, GRIM-19 and ERBIN (*Werts et al.*, 2006). NODs can also activate MAP kinase pathways, for example, NOD2 has been shown to activate p38 and ERKs (*Kobayashi et al.*, 2005), whereas NOD1 was implicated in JNK activation (*Girardin et al.*, 2001).

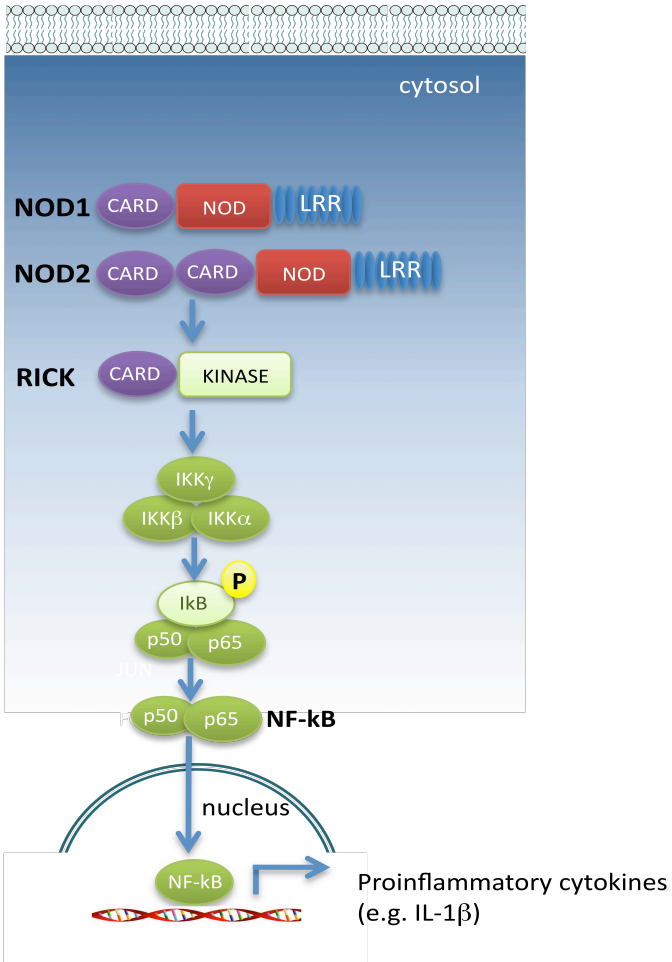


Figure 6 – Major signaling pathway of elicited upon NOD1 and NOD2 stimulation. NOD1 and NOD2 recruit TICK after recognition of their ligands (e.g. iEDAP and MDP, respectively). RICK in turn activates the IkB kinase (IKK) complex and nuclear factor NF-kB sequentially, leading to the translocation of NF-kB to the nucleus and subsequent pro-inflammatory cytokine transcription. Adapted from (*Lee et al.*, 2007).

In what concerns the NALP family of NLRs, several members have been involved in the activation of signal-induced multiprotein complexes called Inflammasomes (*Martinon et al.*, 2002; *Martinon et al.*, 2007a). Inflammasome complexes are involved in the activation of inflammatory Caspases, such as Caspase-1, that are essential for the processing of immature pro-inflammatory cytokines (pro-IL-1β, pro-IL-18 and possibly pro-IL-33) into their mature and secreted forms (reviewed in *Tschopp et al.*, 2003; *Martinon et al.*, 2009). At least two types of NALP Inflammasomes have been identified, the NALP1 and the NALP2/3 Inflammasomes. The NALP1 Inflammasome comprises NALP1, the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD domain), Caspase-1 and Caspase-5 (Figure 7). NALP2/3 Inflammasomes composed of ASC, CARDINAL and Caspase-1, in addition to NALP2 or NALP3 (Figure 7, *Martinon*, 2004; *Martinon et al.*, 2007a). In both Inflammasomes, activated NALPs, via their PYD effector domains, recruit ASC (containing PYD and CARD domains), which in turn recruits Caspase-1 via CARD-CARD domain interaction. NALP1 also recruits Caspase-5 via its additional CARD effector domain at the C-terminus, whereas NALP3, lacking such a CARD domain, can recruit additional Caspase-1 via interaction with the CARD-containing adaptor protein, Cardinal. The close proximity of the Caspases in the Inflammasomes is thought to promote their cross-activation, thus leading to the maturation of the pro-inflammatory cytokines (reviewed in *Martinon et al.*, 2007a; *Martinon et al.*, 2009).

Members of the IPAF/NAIP family of NLRs have also been shown to be involved in the formation of Inflammasomes and therefore Caspase-1 activation, with subsequent IL-1β processing and release (*Franchi et al.*, 2006; *Miao et al.*, 2006; *Sutterwala et al.*, 2009). IPAF Inflammasome have been shown to be important in the generation of an inflammatory response to several pathogens, such

as *S.typhimurium* (*Mariathasan et al.*, 2004; *Franchi et al.*, 2006; *Miao et al.*, 2006), *L. pneumophila* (*Amer et al.*, 2006; *Zamboni et al.*, 2006), S.flexneri (*Suzuki et al.*, 2007) and *P.aeruginosa* (*Franchi et al.*, 2007; *Sutterwala et al.*, 2007). The IPAF Inflammasome composition and role in IL-1β processing is quite similar to the other NALP Inflammasomes, although, since IPAF can interact directly with pro-Caspase-1 through CARD-CARD interaction (Figure 7), the role of the adaptor molecule ASC in IPAF-mediated Caspase-1 activation is unclear (reviewed in *Lee et al.*, 2007; *Martinon et al.*, 2009). More recently, another Inflammasome have been discovered, the AIM2 Inflammasome. This Inflammasome was shown to be activated upon the recognition of cytosolic dsDNA, and to recruit the ASC adaptor molecule to mediate activation of Caspase-1 (*Burckstummer et al.*, 2009; *Fernandes-Alnemri et al.*, 2009a; *Hornung et al.*, 2009; *Roberts et al.*, 2009).

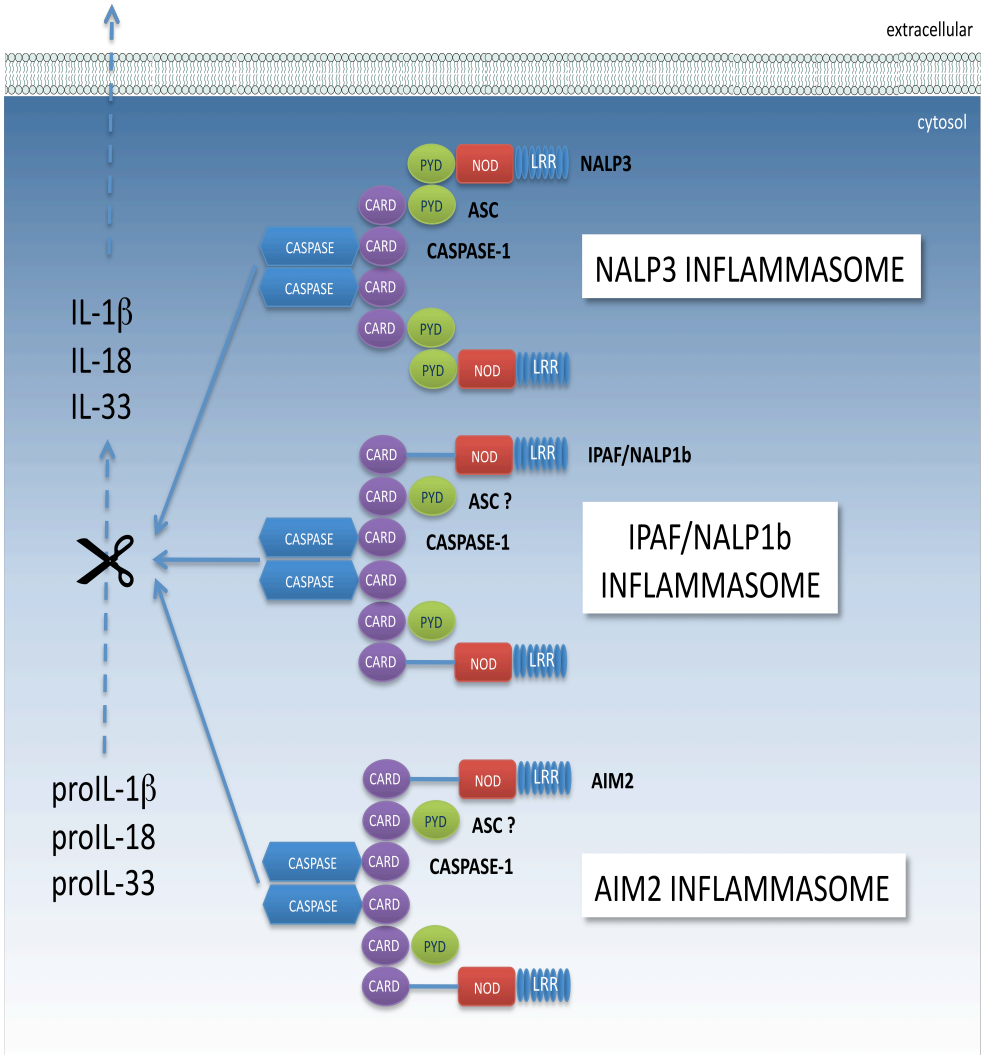


Figure 7 - The different Inflammasomes and their compositions. The Inflammasome name is attributed according to the NLR involved. NALP3 Inflammasome is composed of NALP3, the adapter protein ASC and Caspase-1. Due to the lack of a CARD domain, NALP3 recruits ASC to mediate the interaction with Caspase-1. NALP*1β* and IPAF Inflammasomes composition is still controversial. Although NALP*1β* and IPAF contain a CARD domain, thus the requirement of the adaptor protein ASC to form an Inflammasome is not well defined. Upon Inflammasome activation by the different PAMPS/DAMPs recognized by the NLR, Caspase-1 is activated and processing of proIL-1β, proIL-18 and proIL-33 occurs. The mature forms of the cytokines are then secreted. Adapted from (*Lamkanfi et al.*, 2009a).

1.1.1.2.2.2 - Regulation

The mechanisms of NLR signaling pathways regulation are less understood than the TLR counterparts; however, several regulators of NLRs have also been described (Figure 8). These include ErbB2-interacting protein (ERBIN) that has been shown to bind specifically to NOD2 and therefore inhibiting NOD2 dependent NF-kB and MAPK signaling (*McDonald et al.*, 2005). A short isoform of NOD2, NOD2-s (which encodes only one CARD domain) has been shown to interact with NOD2 adaptor RICK and block NOD2 dependent NF-kB activation (*Rosenstiel et al.*, 2006).

The majority of NLR regulators described so far are related to the Inflammasome regulation, namely the CARD-containing proteins and PYD-containing proteins (reviewed in *Martinon*, 2004; *Stehlik et al.*, 2007; *Martinon et al.*, 2009). The PYD-containing regulators are believed to exert its inhibitory effect due to PYD-PYD interaction between NALPS and the adaptor ASC. Among the PYD regulators are Pyrin, POP1, POP2 and viral PYDs (vPYDs). POPs and some vPYDs (e.g. poxviral gene M13L-PYD, *Benedict et al.*, 2005) are short proteins that contain mainly a PYD domain, therefore the name POP (Pyrin Only Protein). Poxviruses deficient in vPYD show an enhanced activation of Caspase-1 and IL-1β secretion, further demonstrating a negative effect of vPYDs in Inflammasome activation (*Benedict et al.*, 2005; *Johnston et al.*, 2005; *Dorfleutner et al.*, 2007). In a similar way it was shown that POP1 and POP2 modulate Inflammasome activity probably due to ASC-NALP interaction disruption (*Bedoya et al.*, 2007; *Stehlik et al.*, 2007). Pyrin, was initially identified as the product of the MEFV gene, which is mutated in patients with Familial Mediterranean Fever (FMF), was shown to interact with ASC, therefore blocking the recruitment of ASC and Inflammasome formation (*consortium*, 1997; *Chae et al.*, 2003; *Chae et al.*, 2006; *Papin et al.*, 2007). Several CARD-containing proteins have been described to inhibit

processing of IL-1β by preventing recruitment and/or activation of the Caspase by the adaptor ASC, due to homotypical CARD-CARD interactions. Iceberg, a highly similar protein to the CARD of Caspase-1 was shown to function as a decoy protein by sequestering Caspase-1 via its CARD domain, therefore preventing binding to activating adaptors (*Humke et al.*, 2000; *Druilhe et al.*, 2001). Other highly homolog proteins to the Caspase-1 CARD domain have been found to, by a mechanism similar to Iceberg, disrupt Caspase-1 activation, INCA and COP1/Pseudo-ICE respectively (*Druilhe et al.*, 2001; *Lee et al.*, 2001; *Lamkanfi et al.*, 2004). Caspase-12 was recently identified as a negative regulator of the inflammatory cytokine response by binding to and inhibiting Caspase-1 (*Saleh et al.*, 2004; *Saleh et al.*, 2006). The pro-domain (a CARD domain) was shown to be sufficient for causing reduced cytokine secretion. Although, NOD2 precise role in Caspase-1 activation is still very controversial, it has been shown that although NOD2-S does not interact with Caspase-1 but with its adaptor RICK and competes with NOD2 for RICK binding, it results in impaired Caspase-1 activation (*Rosenstiel et al.*, 2006). The NLR protein PYNOD (NALP10) interacts with ASC but lacks the ligand-sensing LRRs (*Wang et al.*, 2004). Although removal of the LRR from NLRs usually renders them constitutively active, PYNOD associates with ASC without recruiting and activating Caspase-1 (*Wang et al.*, 2004). One of the first identified Caspase-1 inhibitors was cytokine response modifier A (CrmA) from cowpox virus, which forms a complex with the catalytic center of Caspase-1 (*Ray et al.*, 1992). Caspase-1 activity can also be blocked by Flightless-I (*Li et al.*, 2008) and the serpin protease inhibitor 9 (Pi9, *Young et al.*, 2000). More recently, Bcl-2 and Bcl-xL were also suggested to influence NALP1 Inflammasome activation (*Bruey et al.*, 2007).

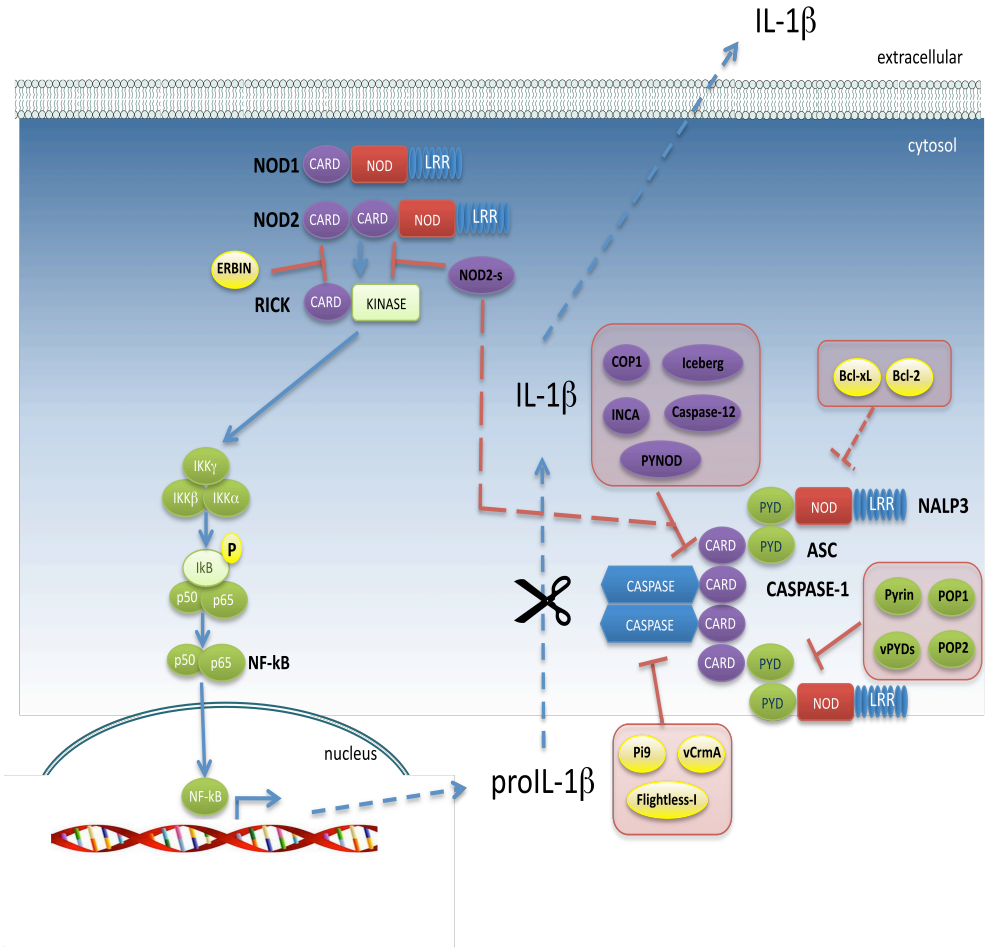


Figure 8 - Negative regulation of NLRs signaling pathways and Inflammasome. NOD signaling is inhibited by ErbB2-interacting protein (ERBIN) and the alternatively spliced isoform of NOD2 (NOD2s), which directly bind to NOD2 or compete with NOD2 for RICK binding, respectively, therefore inhibiting downstream signaling. Several CARD-containing proteins (represented in violet) have been identified to inhibit NALP3 Inflammasome by binding to ASC/Caspase-1 CARD domains. Among them are COP1, INCA, Caspase-12, Iceberg, PYNOD and NOD2s, however this last one is still under debate. PYD-containing proteins (depicted in green) that interact with PYD domains of NALP3 and ASC have also been reported to inhibit the Inflammasome. These include Pyrin, viral PYD-containing proteins (vPYDs), POP1 and POP2. Caspase-1 can be directly blocked by Flightless-I, the serpin protease inhibitor (Pi9) and the cowpox-virus encoded inhibitor of Caspase-1, vCrmA. Bcl-2 and Bcl-xl proteins have been shown to inhibit NALP1 Inflammasome oligomerization, by binding to NALP1. Adapted from (*Martinon et al.*, 2009).

1.1.2 - The IL-1β pathway: a PRR crosstalk

Several types of PRR-PRR interplay have already been described. Many TLRs can influence the expression of other TLRs. For example, TLR3 and 4 stimulation was shown to upregulate TLR2 expression on the surface of macrophages in a MyD88-independent manner, whereas TLRs 2, 7 and 9 do so in a MyD88-dependent way (*Nilsen et al.*, 2004). TLR4 stimulation can upregulate TLRs 2, 4 and 9 mRNA expressions in DCs, in an ERK and NF-kB dependent manner (*An et al.*, 2002a; *An et al.*, 2002b). Although this upregulation of TLRs by other TLRs stimulation often results in an increased Immune response due to the engagement of several TLRs, the initial stimulation dose and the timing of stimulation by the second TLR agonist can influence the Immune response (*Napolitani et al.*, 2005). For example, it has been shown that a strong first stimulation of the TLR can also induce TLR-signaling inhibitors expression, such as MyD88s (a short splice variant of MyD88 that acts as a dominant negative isoform of MyD88 dependent signaling pathway, *Janssens et al.*, 2002).

Certain TLRs can also diversify their recognition repertoire by interacting with other TLR/PRRs. As an example, TLR2 can form heterodimers with TLR6 and TLR1 to recognize different lipopeptides (as shown in Figure 2), dyacil and tryacil lipopeptides, respectively (*Takeuchi et al.*, 2001; *Takeuchi et al.*, 2002). Furthermore, TLR2/6 complex can also interact with other PRRs, such as Dectin-1 (a member of the C-type lectin family involved in the recognition of fungal cell wall components, such as β-glucans, *Brown et al.*, 2001; *Pyz et al.*, 2006) to recognize microbes and therefore to modulate an Immune response (*Gantner et al.*, 2003; *Brown*, 2006; *Dillon et al.*, 2006).

Interplay between TLRs and NODs has also been described. For example, NOD1 and NOD2 agonists act cooperatively with LPS (a typical TLR4 agonist) to stimulate the production of inflammatory cytokines such as TNFα and IL-6, induce DC maturation (*Fritz et al.*, 2005) and T cell differentiation (*Tada et al.*, 2005). However the underlying mechanism(s) for the synergistic effect of NOD agonists on TLR-mediated cytokine production is largely not well understood. There are already some "hints" such as the increased expression of NOD2 and RICK upon TLR4 stimulation (*Inohara et al.*, 2005) and the upregulation of MyD88 and TAK1 expression upon NOD2 and NOD1 stimulation, respectively (*Inohara et al.*, 2005; *Masumoto et al.*, 2006). The classical example, and probably the best-studied crosstalk between TLRs and other PRRs, is their joint action with NLRs leading to the secretion of mature IL-1β in an inflammatory response (*Creagh et al.*, 2006; *Dinarello*, 2009; *Martinon et al.*, 2009).

1.1.2.1 - The IL-1β pathway

Interleukin-1β secretion is mainly described as a two-step process, that requires production of immature pro-IL-1β and a second processing step that leads to the maturation of this cytokine, prior to its release (reviewed in *Eder*, 2009; *Dinarello*, 2009; *Martinon et al.*, 2009; *Netea et al.*, 2010). While the first step required for IL-1β secretion, the production, is mainly TLR activation dependent, the second and processing step is NLR dependant (Figure 9, reviewed in *Eder*, 2009; *Dinarello*, 2009). After stimulation, TLRs induce activation of the NF-kB pathway, leading to a strong IL-1β transcription (reviewed in *Creagh et al.*, 2006; *Lee et al.*, 2007; *Dinarello*, 2009; *Martinon et al.*, 2009). However, several exceptions were already reported, as members of the NOD subclass of NLRs were also identified as important mediators of IL-1β production following different

stimuli, such as intracellular bacteria (Figures 6 and 9 , reviewed in *Chamaillard et al.*, 2003; *Delbridge et al.*, 2007; *Franchi et al.*, 2007; *Lee et al.*, 2007). The translation step, that generates the immature pro-IL-1β occurs in the cytosol. Then, the processing, that leads to the effective production of mature IL-1β and its secretion depends on the activation of the Inflammasome complex (Figure 9, *Martinon et al.*, 2002; reviewed in *Martinon et al.*, 2009). Several major Inflammasomes involved in the activation of Caspase-1 and subsequent IL-1β processing have been described, but the NALP3 Inflammasome is by far the most studied (Figure 7, reviewed in *Lamkanfi et al.*, 2009; *Martinon et al.*, 2009). A large number of stimuli have been described to activate the NALP3 Inflammasome, as described in Table 2, however the precise mechanism that leads to its activation is still unclear and several hypothesis have been drawn. A direct ligand-receptor interaction, between the LRRs of NALP3 and the activator, have been conceptually proposed to occur. This interaction would lead to a conformational change in the NLR, allowing oligomerization and Inflammasome formation, however no direct binding between NALP3 activators and the NLR itself have been found (reviewed in *Martinon et al.*, 2009; *Stutz et al.*, 2009). Another hypothesis relies on the necessity of low intracellular potassium concentrations to promote NALP3 Inflammasome activation (*Pétrilli et al.*, 2007). This hypothesis is based in the evidences showing a need for potassium efflux for the activity of most, if not all, NALP3 Inflammasome stimuli (e.g. pore forming toxins, *Perregaux et al.*, 1994; *Walev et al.*, 1995; *Pétrilli et al.*, 2007; *Dostert et al.*, 2008). Others claim that ROS can act upstream of the NALP3 Inflammasome, therefore indirectly causing its activation (*Cruz et al.*, 2007; *Pétrilli et al.*, 2007; reviewed in *Stutz et al.*, 2009). Phagosomal and lysossomal destabilization phenomenon as a mechanism of Inflammasome activation has also been proposed. Activation of NALP3 Inflammasome was observed upon phagosomal destabilization using crystals (e.g. silica) or by pharmacological disruption of

lysosomes (*Hornung et al.*, 2008). According to this theory, NALP3 can sense phagosomal or lysosomal disruption as danger signals and thereby could indirectly sense excessive crystal phagocytosis or escape of microbes from the lysosomal compartments to the cytosol, therefore activating the processing and release of this pro-inflammatory cytokine (reviewed in *Stutz et al.*, 2009; *Shi et al.*, 2010).

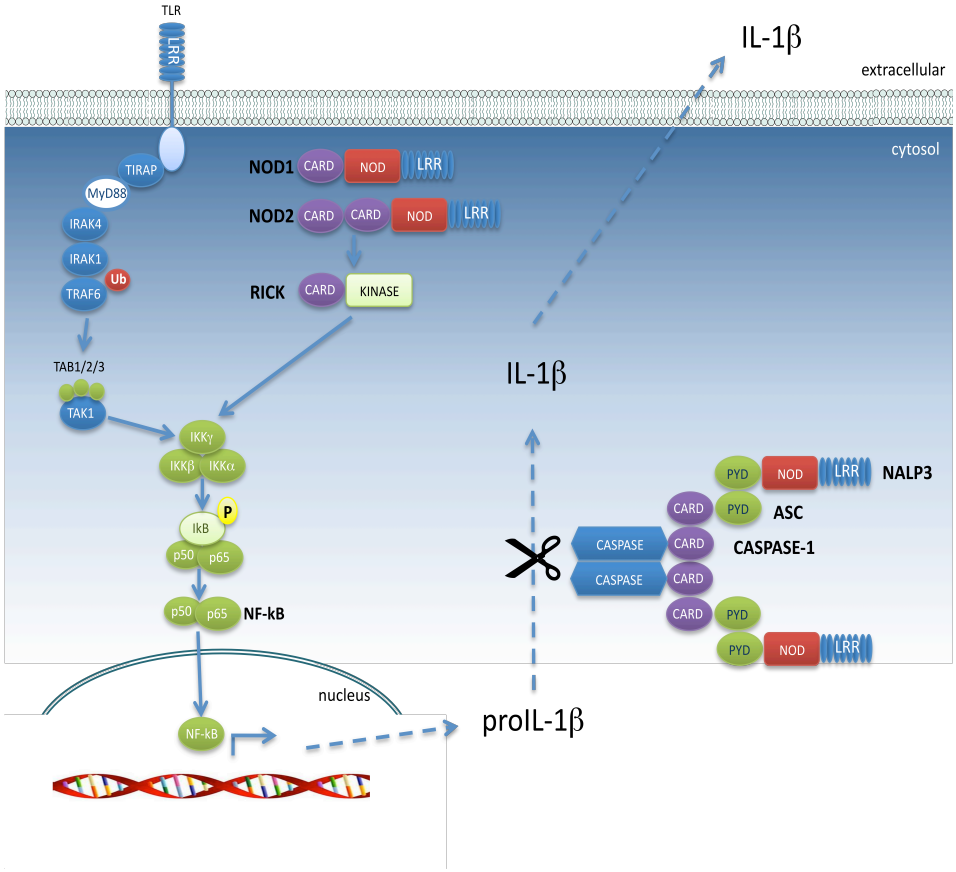


Figure 9 – IL-1β synthesis and processing, a classical example of TLRs and NLRs interplay. TLR stimulation induces strong transcription of IL-1β via activation of the NF-kB transcription factor. NOD1 and NOD2 stimulation can also activate NF-kB pathway and therefore IL-1β transcription. IL-1β is produced as an inactive cytoplasmic precursor that is proteolytic processed by the inflammatory Caspase-1 to generate the mature secreted active form. Caspase-1 is also synthesized as an inactive form that requires processing by the Inflammasome to become active. Upon activation of NALP/IPAF NLR (e.g. NALP3) the Inflammasome complex is formed, and Caspase-1 activated, leading to the sequential maturation of IL-1β into its active secreted form. Adapted from (*Lee et al.*, 2007).

The importance of TLRs in IL-1β pathway does not seem to be restricted to the production of a large quantity of the immature proIL-1β protein, it is far more complex than that. Several evidences suggest the involvement of TLR signaling in Caspase-1 activation itself. Costimulation of macrophages with extracellular ATP (a known inducer of potassium efflux) and LPS (TLR4 agonist) activates Caspase-1 efficiently, whereas extracellular ATP alone does not (*Kahlenberg et al.*, 2005; *Ferrari et al.*, 2006; *Mariathasan et al.*, 2006). *Kahlenberg et al.* have shown that Caspase-1 activation by TLR is mediated by yet unknown protein(s) expressed as a result of NF-kB pathway activation upon TLR stimulation, but not of other signaling pathways such as MAPKs or PI-3K (*Kahlenberg et al.*, 2005). In this context, activation of the NF-kB pathway by different ligands seems to be a critical checkpoint needed for cell priming prior to NALP3 Inflammasome activation by ATP (*Bauernfeind et al.*, 2009; *Franchi et al.*, 2009).

Upon secretion, cytokines elicit their responses by binding to specific high affinity cell-surface receptors on target cells, initiating a series of intracellular signal transduction pathways. Interleukin-1β signals via the Type I IL-1 Receptor (IL-1RI, *Sims et al.*, 1988; reviewed in *O'Neill*, 2008). The IL-1RI is a trasmembrane receptor, composed of three immunoglobulin domains in the extracellular part and a cytoplasmatic signaling TIR domain (reviewed in *Gabay et al.*, 2010). The TIR domain shares some homology with the intracellular domain of the TLR family members, thus, eliciting similar signaling pathways (reviewed in *O'Neill*, 2000; *O'Neill*, 2008). Briefly, upon IL-1β binding, the IL-1R recruits the IL-1R accessory protein (IL-1RacP, *Greenfeder et al.*, 1995). Then, in a similar way to the TLR signaling pathway, successive signaling events will culminate in transcription activation of several genes, mainly in an NF-kB dependent manner (*Osborn et al.*, 1989; reviewed in *O'Neill*, 2000; *O'Neill*, 2008).

1.1.3 - IL-1β (Dis)Regulation and Related Diseases

It is well established that a controlled inflammatory as response to infection or tissue damage is beneficial, however if not tightly regulated can show remarkable detrimental effects (reviewed in *Medzhitov*, 2008). As an example, the induction of IL-1β secretion shows remarkable beneficial effects against infection from a serious number of microbia, however, if not regulated, excessive circulant levels of IL-1β can be the cause of several autoinflammatory diseases (reviewed in *Church et al.*, 2008; *Martinon et al.*, 2009). Increased IL-1β levels have been related to atherosclerosis (*Iida et al.*, 2009), type-2 Diabetes (*Alexandraki et al.*, 2006) and various autoimmune disorders, such as Crohn's disease (*Martinon et al.*, 2009). Neurodegenerative diseases that are accompanied by inflammatory processes, such as Multiple Sclerosis, Alzheimer or Parkinson's diseases, have also been characterized by augmented IL-1β levels (*Allan et al.*, 2005; *Simi et al.*, 2007). Interleukin-1β has also been shown to facilitate tumor invasiveness (*Apte et al.*, 2006) and promote tissue damage in the pathogenesis of a chronic Inflammation (*Church et al.*, 2008; *Martinon et al.*, 2009). Blocking IL-1β receptor with synthetic IL-1β receptor antagonists, such as Anankira (*Fitzgerald et al.*, 2005; *Kalliolias et al.*, 2008; *Lequerre et al.*, 2008), was found to reduce the severity of systemic-onset juvenile arthritis, acute pancreatitis and several autoinflammatory disorders, including the adult-onset Still's disease, lupus arthritis, among others (*Dinarello*, 2005b; *Burger et al.*, 2006; *Martinon et al.*, 2009).

The important role of the Inflammasome and IL-1β in the inflammatory process is strongly supported by the genetic evidences that link the Inflammasome misregulation to several disorders, such as Family of Hereditary Periodic Fevers (HPFs, *McGonagle et al.*, 2007; reviewed in *Church et al.*, 2008; *Stutz et al.*,

2009). Among the best described HPFs, is the cryopyrinopathy subfamily that include the Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS) and the Chronic Infantile Neurological Cutaneous and Auricular Syndrome (CINCA/NOMID). All of these disorders are due to mutations in the third exon of the NALP3 gene, which lead to spontaneous oligomerization of NALP3 Inflammasome with subsequent Caspase-1 activation, giving rise to excessive and inappropriate IL-1β release (*Agostini et al.*, 2004; *Aksentijevich et al.*, 2007; *Shinkai et al.*, 2008). Other autoinflammatory diseases, such as FMF, have also been associated with the NALP3 Inflammasome misregulation. FMF is an autossomal-recessive disorder caused by mutation in the Pyrin gene, leading to a reduced ability to moderate IL-1β processing and release (*Gumucio et al.*, 2002; *Papin et al.*, 2007). Mutations in other NLRs have also been found to be the causative effect or to increased susceptibility to several inflammatory diseases (such as Crohn's disease and Hereditary Fever Syndrome, reviewed in *McGonagle et al.*, 2007; *Martinon et al.*, 2009; *Stutz et al.*, 2009; *Gabay et al.*, 2010). Mutated NOD2 gene have been found to be associated with increased susceptibility to Crohn's disease, a chronic and relapsing inflammatory disease of the bowel (*Ogura et al.*, 2001, *Maeda et al.*, 2005), while several mutations in the NALP12 gene have been associated with hereditary periodic fever syndromes (*Jéru et al.*, 2008).

Because of its potency, extensive functions and the increased number of diseases associated with excessive circulant levels of IL-1β, it became clear the necessity of a tight regulated mechanism of IL-1β secretion in order to avoid excessive inflammatory conditions leading to disease.

The process that leads to IL-1β secretion is the outcome of a two-step mechanism where TLRs are involved primarly in the first step and NLRs in the second. Consequently, all the regulators mentioned reported previously to be involved in the regulation of either TLR or NLR signaling pathways, can exert

similar functions in the regulation of IL-1β secretion. As an example, known inhibitors of the Inflammasome, such as Iceberg or Pyrin, were shown to be able to modulate IL-1β secretion (*consortium*, 1997; *Humke et al.*, 2000; *Druilhe et al.*, 2001; *Chae et al.*, 2003; *Chae et al.*, 2006; *Papin et al.*, 2007). In a similar manner, known regulators of TLR signaling pathway were shown to be able to regulate the secretion of this cytokine (e.g. MyD88s, *Janssens et al.*, 2002).

1.1.3.1 - Alternative Splicing and the Regulation of IL-1β secretion

A functional Immune system utilizes a wide range of proteins produced by Immune cells to adapt and respond to the rapidly changing environmental conditions. Thus, the massive functional changes required to respond to the diverse challenges the Immune System has to face, several mechanisms of gene regulation are required to achieve diversity and flexibility of functions (*Lynch*, 2004; *Miosge et al.*, 2005). Alternative Splicing (AS) has emerged recently as a major regulatory process that contributes to the biological complexity and diversity required by a competent Immune system. Alternative Splicing can direct the inclusion or exclusion of regulatory sequences located in the 5' or 3' untranslated regions, therefore capable of controlling mRNA stability, cellular distribution and translation efficiency (Chapter 1.2, reviewed in *Black, 2003; Chen et al., 2009*). In addition, AS can alter the structure of the gene product by the insertion or deletion of novel protein coding parts, therefore allowing a single gene to generate more than one messenger RNA molecule (mRNA), so-called isoforms (reviewed in *Black, 2003; Wang et al., 2008; Chen et al., 2009)*. As a matter of fact, an increasing amount of studies focus AS as one of the most important protein

diversity generator mechanisms involved in the regulation of several cellular functions, where Immune responses are obviously included (reviewed in *Black*, 2000; *Lynch*, 2004; *Leeman et al.*, 2008; *Wang et al.*, 2008; *Sahoo et al.*, 2010).

In the past years, in an attempt to unveil the mechanisms involved in the regulation of Inflammation and other Immune responses, diverse isoforms of important key-molecules in different signalling pathways were found to recurrently be involved in their regulation (reviewed in *Lynch*, 2004; *Litman et al.*, 2007; *Leeman et al.*, 2008; *Mourich et al., 2009*). As an example, splice variants have been detected in several genes expressed in T cells, with functional consequences (reviewed in *Lynch*, 2004). One example is the Splicing of the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), where either a transmembrane or soluble forms are generated by AS in response to T cell activation (*Magistrelli et al.*, 1999; *Oaks et al.*, 2000). Another example is the Splicing of protein tyrosine phosphatase CD45. It was shown that different AS generated isoforms of CD45 are present in naïve and active T cells (*Trowbridge et al.*, 1994; *Lynch et al.*, 2000; *Oberdoerffer et al.*, 2008).

Several genes involved in IL-1β secretion and Inflammation have also been shown to be regulated by AS (Figure 10, reviewed in *Leeman et al., 2008; Krieg et al., 2009; Matsushita et al., 2009a; Sahoo et al., 2010*). An Alternatively spliced isoform of the MyD88, MyD88s, was reported to be able to modulate TLR signaling pathway activation and IL-1β secretion (*Janssens*, 2002). This short isoform was reported to be induced in monocytes after 16 hr of LPS challenge. Despite the capabillity of MyD88s to bind the IL-1R and IRAK, it is defective in its ability to induce IRAK phosphorylation and NF-κB activation, therefore behaving as a dominant negative inhibitor of IL-1β secretion upon LPS challenge (*Janssens*, 2002; *Burns et al.*, 2003; *Janssens*, 2003). Similar to MyD88s, alternatively spliced isoforms of other important molecules involved in the IL-1β pathway have been found to be able to regulate this pathway, such as: NOD2s, a

short isoform of NOD2 (*Rosenstiel et al.*, 2006; *Leung et al.*, 2007); IRAK2 isoforms (*Hardy et al.*, 2004) and more recently an ASC isoform was also identified, vASC, also capable of modulating IL-1β secretion (*Matsushita et al.*, 2009a). Alternative Splicing isoforms of the IL-1R antagonist (IL1-RA) were also reported to exist, namely a secreted (sIL-1RA) and two intracellular (icIL-1RaI, icIL-1RaII) isoforms (*Malyak et al.*, 1998; reviewed in *Sahoo et al.*, 2010). The endogenous expression or exogenous administred IL-1RA isoforms antagonize the IL-1β pro-inflammatory effects, therefore being capable of modulating this Immune response (reviewed in *Dinarello*, 2009). The expression of IL-1β and its receptor, IL-1R was also demonstrated to be regulated by AS (reviewed in *Sahoo et al.*, 2010). Three isoforms of IL-1RI were reported in primary cultures of rat hepatocytes. Two of the membranar isoforms were related to increased transcription and mRNA stability of the inducible Nitric Oxide Synthase, whereas the soluble isoform decreased the IL-1β induced Nitric Oxide production (*Yamada et al.*, 2007). Several isoforms of IL-1β were identified in different cell types and organisms. In rainbow trout, three IL-1β isoforms of incompletely spliced variants, containing intron 5 or introns 4 and 5, were detected in gills, kidney, liver and spleen tissue from bacterially challenged fish but only the fully spliced transcript was detectable in blood (*Sahoo et al.*, 2010). In equine peripheral blood mononuclear cells, an isoform of IL-1β not containing the exon 5 region necessary for normal maturation of the cytokine by Caspase-1 was also identified (*Kato et al.*, 1996). Moreover, the expression of the different IL-1β isoforms is sometimes linked with the appearance of disease. For example, increased expression of the IL-1β splice variant 1 (IL-1βsv1) in the myocardium of dogs is linked with a severe heart failure due to a chronic degenerative valvular disease (*Kiczak et al.*, 2008).

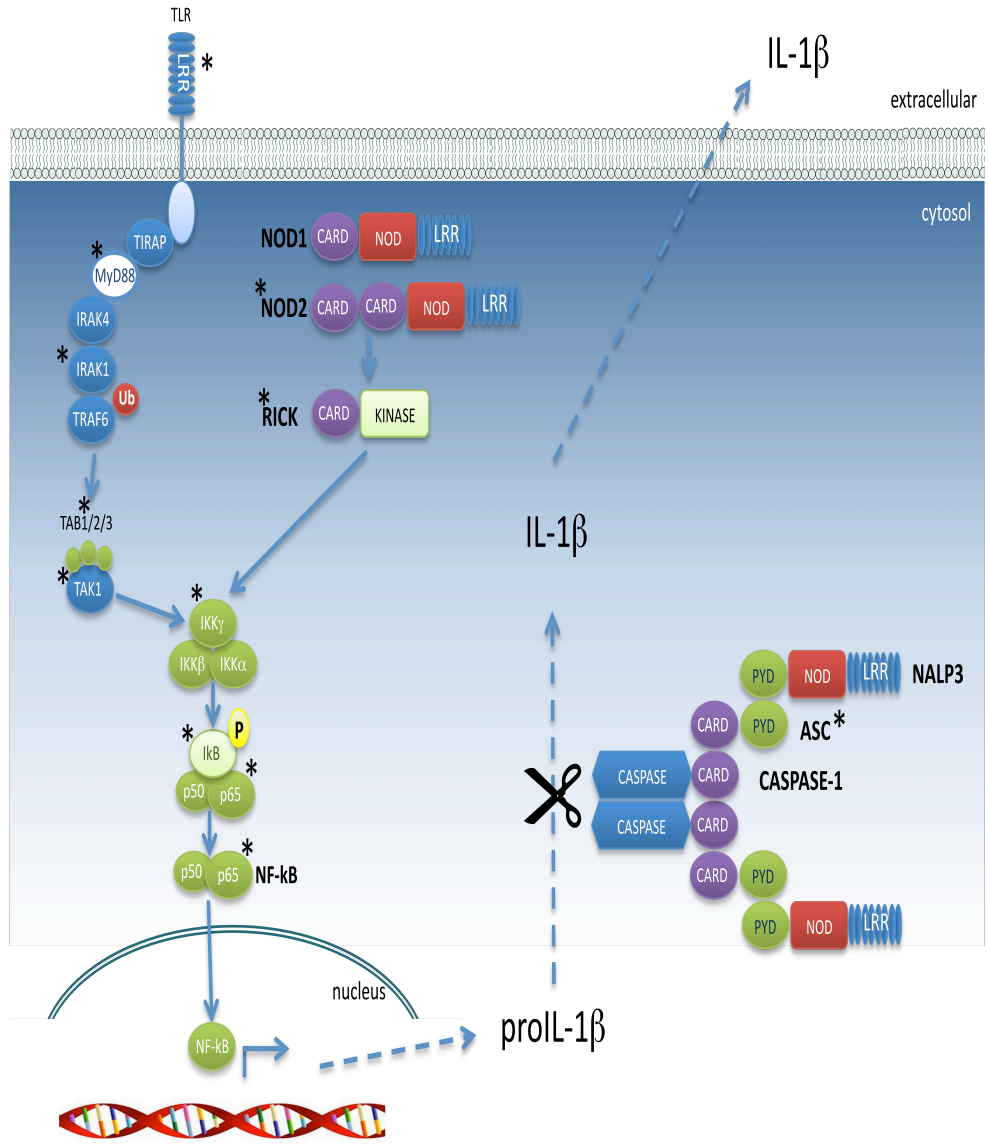


Figure 10 - Alternative Splicing regulation of IL-1β secretion. Several genes involved in the regulation of IL-1β secretion pathway have been shown to be regulated by AS. These genes are marked with an asterisk. Adapted from (*Leeman et al.*, 2008; *Krieg et al.*, 2009; *Matsushita et al.*, 2009a).

In sum, as represented in Figure 10, the expression of a large number of genes involved in IL-1β secretion pathway were already described to be regulated by AS. Despite the identification of the different isoforms of important molecules involved in this pathway, not much is known about the mechanisms involved in the regulation of their expression and their roles in the regulation of IL-1β secretion. All these information lead us to investigate impact of AS in the regulation of Inflammation, namely the regulation of IL-1β secretion.

Chapter 1.2 - Alternative Splicing

"*A number of genes in higher organisms and in their viruses appear to be split… they have nonsense stretches of DNA interspersed within the sense DNA. The cell produces a full RNA transcript of this DNA…and then appears to splice out the nonsense sequences before sending the RNA to the cytoplasm*."

Francis Crick, 1979

In Eukaryotes, gene expression involves several steps that start with transcription. After transcription, where DNA is transcribed into a precursor messenger RNA (pre-mRNA) by the RNA polymerases (RNA pol), several processing steps (e.g. 5'capping, Splicing, 3'end processing and editing) occur in the cell nucleus before the export of the mature messenger RNA (mRNA) to the cytoplasm, where it serves as template for the synthesis of a protein (reviewed in *Orphanides et al.*, 2002). However, it is now accepted that some of these steps occur simultaneously as a part of a continuous process, and not in an independent successive mechanism as it was thought in the past (reviewed in *Cramer et al.*, 2001; *Orphanides et al.*, 2002; *Licatalosi et al.*, 2010). For example, coupling between transcription and the capping step of pre-mRNA processing (reviewed in *Shatkin et al., 2000; Cramer et al., 2001; Proudfoot et al., 2002*) and between transcription and pre-mRNA Splicing (reviewed in *Cramer et al., 2001; Fong et al., 2001; Kornblihtt et al., 2004).*

Several RNA polymerases were identified, however, the majority of eukaryotic genes are transcribed by RNA pol II transcription machinery (reviewed in *Cramer et al., 2001)*. The primary transcripts synthesized by the RNA pol II, pre-mRNA, undergo several specific modification steps before being transported to the cytoplasm, namely: 5'capping, Splicing, 3' processing and editing (reviewed in *Cramer et al., 2001; Orphanides et al.*, 2002).

A capping step at the 5' end occurs soon after the RNA poll II transcribes the first 25-30 nucleotides (reviewed in *Proudfoot et al., 2002)*. This 5' cap minimizes its degradation by the nucleases and allows the interaction with proteins involved in export of mRNA into the cytoplasm (reviewed in *Cramer et al., 2001;Proudfoot et al., 2002).*

The pre-mRNA Splicing is an essential step in the regulation of gene expression due to the nature of eukaryotic genes. An eukaryotic gene is characterized by a very large interruption of the coding sequence with large non-coding sequences, which have to be removed during this step of RNA biogenesis in order to place the coding sequences in a protein-reading frame (discussed further in greater detail, reviewed in *Orphanides et al.*, 2002; *Soller*, 2006).

At the 3'end, the majority of RNA pol II transcripts are cleaved and a polyadenosine, poly(A), tail of about 200-250 adenosines is added, providing binding sites for a major class of regulatory factors, the poly(A)-binding proteins (PABPs,*Orphanides et al.*, 2002; *Soller*, 2006). These PABPs exert several function in the gene expression pathway, such as: polyadenylation, mRNA export, translation and turnover of the transcripts to which they are bound (reviewed in *Mangus et al.*, 2003).

Until recently, the step of RNA editing was known to occur only in a few number of transcripts and was largely understimated due to the difficulty to detect the editing sites (reviewed in *Skarda et al.*, 2009). This step is characterized by single nucleotides changes at the pre-mRNA level, either insertions or deletions of

nucleotides or by nucleic acid bases substitutions. These modifications can affect both coding and non-coding sequences, therefore this editing event can change both the sequence and the secondary structure of RNA molecules, with important consequences for both the final proteins and regulatory RNAs (reviewed in *Keegan et al., 2001; Skarda et al., 2009).*

Once processed, the mRNA is exported by binding to factors in the nucleus that direct the mRNA molecules into the cytoplasm through interactions with proteins that line the nuclear pores (reviewed in *Reed et al.*, 2002; *Cole et al.*, 2006b; *Cole et al.*, 2006a; *Carmody et al.*, 2009).

In the cytosol, protein biosynthesis, or so-called translation, occurs on the ribosome, a large RNA-protein complex (reviewed in *Ramakrishnan*, 2002; *Dunkle et al.*, 2010; *Jackson et al.*, 2010). The mRNA translation, is a highly coordinated mechanism involving many proteins and RNAs, which is generally divided into three steps: initiation, elongation, and termination. Translation initiation can be subdivided into three steps: the binding of the specific start codon (AUG) ribosomal subunit; binding of the resulting complex to an mRNA and locating the initiation codon; and third, joining of the large ribosomal subunit to generate a translation-competent ribosome (reviewed in *Ramakrishnan, 2002; Sonenberg et al., 2003; Dunkle et al., 2010; Jackson et al., 2010*). The elongation process is a much simpler step that only requires to maintain the reading frame, select and deliver the correct aminoacyl-tRNAs to the ribosome, and form peptide bonds (reviewed in *Groppo et al.*, 2009; *Van Der Kelen et al.*, 2009). Upon the recognition of the stop codons, UAA, UAG or UGA, translation is termined (reviewed in *Groppo et al.*, 2009; *Van Der Kelen et al.*, 2009). Then, the nascent polypeptide undergoes folding and post-translational modification to give rise to the final active protein (reviewed in *Daggett et al.*, 2003; *Groppo et al.*, 2009).

1.2.1 - Splicing

The term "Splicing" was probably used for the first time in the 1970s, when *Francis Crick* defined Splicing as the "mechanism by which a single functional RNA molecule is produced by the removal of one or more internal stretches of DNA during the processing of the primary transcript" (Figure 11, *Crick*, 1979). The Splicing discovery was achieved when a simple comparison of the mRNA sequence and its corresponding nuclear DNA revealed that long stretches of DNA were removed during the processing of the longer precursor (*Berget et al.*, 1977; *Chow et al.*, 1977). *Gilbert et al.* defined these long stretches of "DNA" not included in the final mRNA as "introns" (intervening sequences), and named "exons" (expressed sequences) the sequences of "DNA" that end up expressed in the final mRNA (*Gilbert*, 1978). Nowadays, it is known that the process of intron removal occurs at an RNA processing step and not at the DNA level, as suggested by *Gilbert*. However, since then, the terminology intron/exon is still used to define those regions excluded and included in the mRNA, respectively (reviewed in *Black*, 2003).

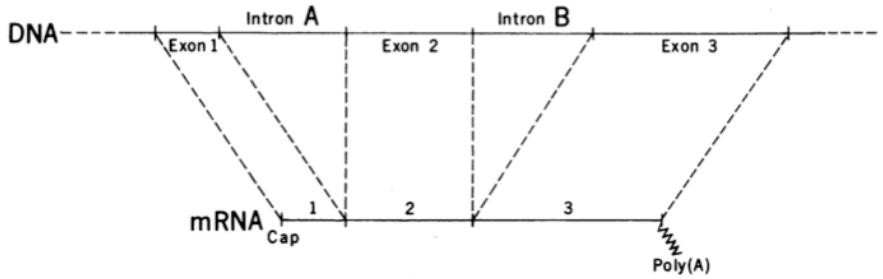


Figure 11 - RNA Splicing schematics. The gene is encoded by 3 exons (1, 2 and 3) and 2 introns (A and B). In the final mRNA just the exons are expressed and the introns were spliced out. Adapted from (*Crick*, 1979).

In the early times of RNA Splicing studies, four mechanisms of Splicing were proposed. First, the DNA rearrangement mechanism, where the cell would rearrange its own DNA in order to displace or eliminate the sequences that were not needed. Second, the process where DNA would remain unaltered, but the RNA polymerase would "jump" along the DNA sequence, skipping the introns and including only the exons in the mature RNA. The third mechanism proposed that each exon would be transcribed separately, and the pieces joined together to form the mature RNA. Finally, the fourth mechanism where the RNA polymerase would make a primary transcript of the whole region, including exons and introns. This transcript would then be processed so that the introns were removed and the exons joined together (reviewed in *Crick*, 1979). Nowadays, it is accepted that the Splicing mechanism occurs in a two-step fashion (Figure 12), both involving single transterification reactions. In the first step, the 2'-hydroxyl group of an A residue at the branch point attacks the phosphate at the 5' splice site. This process will lead to the cleavage of the 5' exon from the intron and the concerted ligation of the intron 5' end to the branch point 2'-hydroxyl, producing two reaction intermediates, a detached 5' exon and an intron/3'-exon fragment in a lariat configuration containing a branched A nucleotide at the branch point. The second transterification reaction is the attack on the phosphate at the 3'end of the intron by the hydroxyl of the detached exon. This ligates the two exons and releases the intron, still in the form of a lariat (reviewed in *Black*, 2003).

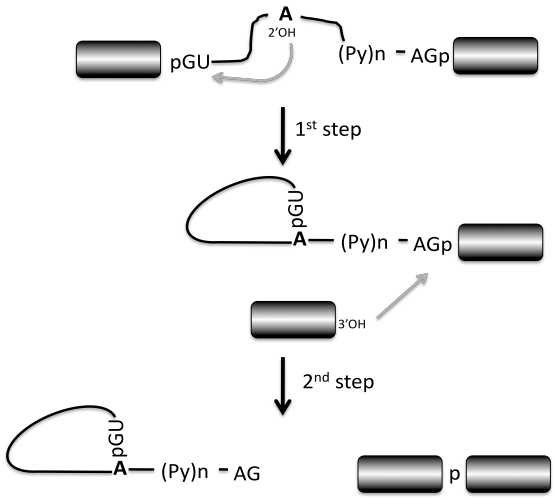


Figure 12 – **Splicing reaction**. The Splicing reaction takes place in two transterification steps. The first step results in two reaction intermediates: the detached 5'exon and an intron/3' exon fragment in a lariat structure. The second step ligates the two exons and releases the intron lariat. Adapted from (*Burge*, 1999).

1.2.1.1 - At the Splicing Locus

The RNA Splicing, like other events of gene expression is tightly regulated. Pre-mRNA Splicing regulation is achieved by the interplay between the *cis*-acting RNA sequence elements, and protein and/or RNA *trans*-acting factors that recognize these sequences. The position and composition of the *cis*-acting elements help to define the splice sites through the recruitment of the Spliceosome complex (*Smith et al.*, 2000; *Black*, 2003). The Spliceosome is normally defined as a large multiprotein complex composed of five small nuclear ribonucleoproteins (snRNPs, U1, U2, U4, U5 and U6) and more than 50 non-snRNPs that recognize and assemble on the exon-intron processing of the pre-mRNA (reviewed in *Makarov et al.*, 2002; *Black*, 2003; *Jurica et al.*, 2003)

1.2.1.1.1 - Splicing Machinery - The Spliceosome assembly and its dynamics

The Spliceosome complex is not static, it is a highly dynamic molecular machinery whose composition is also not static and whose structure undergoes multiple rearrangements during each Splicing cycle. Spliceosome assembles onto the pre-mRNA through a series of complexes (Figure 13). During the assembly, the U1 snRNP binds to the 5' splice site via base pairing between the splice site and the U1 snRNA, whereas the 3' splice site elements are initially bound by a special set of proteins. SF1/BBP binds to the branch point (A) and the U2 snRNP auxiliary factor (U2AF), a heterodimer composed of a large ($U2AF^{65}$) and a small ($U2AF^{35}$) subunits, binds to the polypyrimidie tract (Py). The earliest defined complex in Spliceosome assembly, the E complex, contains the U1 and U2AF bound at the two intron ends. The E complex is then joined by the U2 snRNP, whose snRNA base pairs at the branch point, to form the A complex. This A complex then recruits the pre-formed U4/U6.U5 tri-snRNP, to form the mature Spliceosome, also called complex B. The conversion of the mature Spliceosome into a complex capable of catalyzing Splicing, named the activated Spliceosome or complex B*, unveils a major structural change in which the U1 snRNP interaction at the 5' splice site is replaced with the U6 snRNP and both the U1 and U4 snRNPs dissociate from the complex. Subsequently, the activated Spliceosome catalyzes the first transesterification step of Splicing and complex C is formed. After the second step of Splicing, the mRNA is released and the post-spliceosomal complex, containing the excised intron and the U2, U5 and U6 snRNPs, disassembles. The snRNPs are then recycled for new rounds of Splicing (reviewed in *Makarov et al.*, 2002; *Black*, 2003; *Jurica et al.*, 2003).

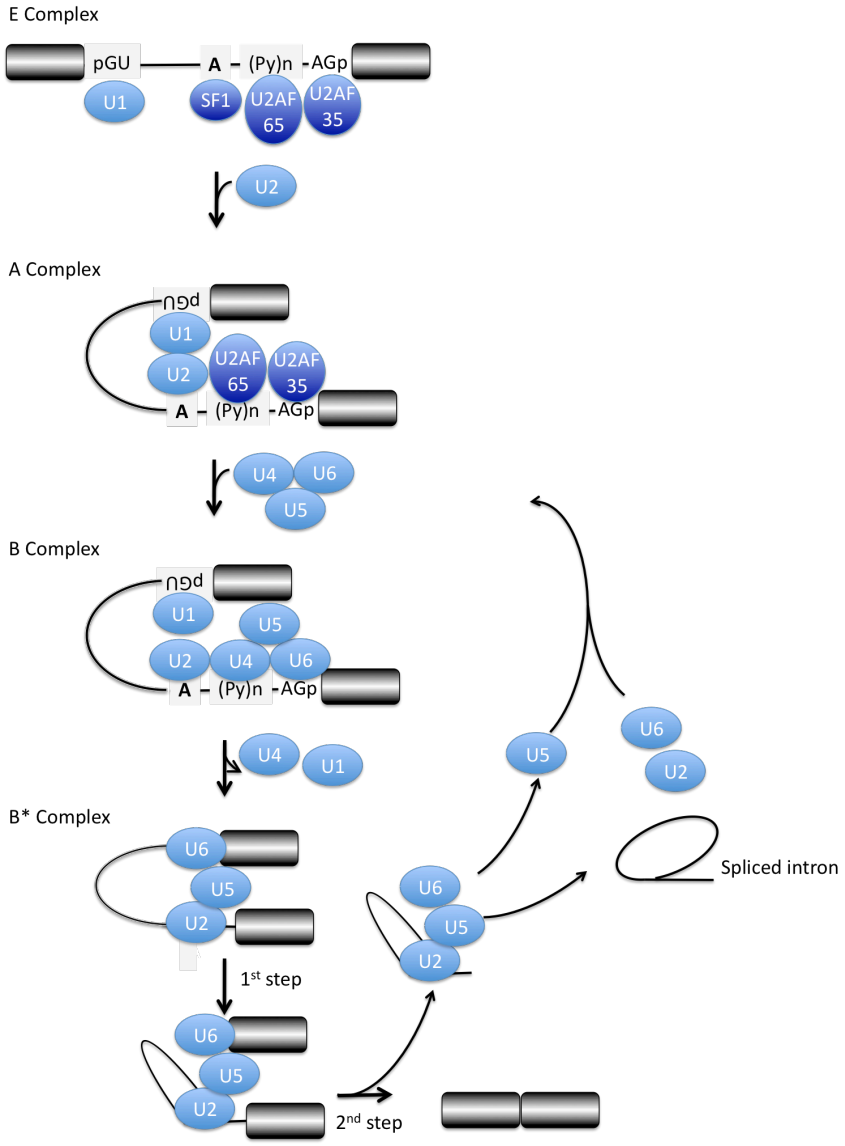


Figure 13 – Spliceosome assembly and dynamics. The Spliceosome contains five small nuclear ribonucleoproteins (U1, U2, U4, U5 and U6) that assemble to the intron (light blue). The presence of auxiliary non-snRNPs is required for Spliceosome assembly and intron removal (represented in dark blue). Adapted from (*Makarov et al.*, 2002; *Black*, 2003; *Jurica et al.*, 2003).

1.2.1.1.2 - Splice site choice - The exon/intron definition

The regulation of Splicing involves both *cis* and *trans* "players', which are sequences in the pre-mRNA and cellular factors (RNA or protein), respectively. The relative functional strength of the splice sites influences the frequency of which an exon is included or excluded. Although, a pair of "strong" splice sites is not sufficient to define an exon. Many pseudo-exons that are flanked by predicted splice sites are not spliced at all (*Sun et al.*, 2000; *Wang et al.*, 2008b). This rises the point that other factors are important in exon definition. Additional elements known as Silencers or Enhancers have been shown to be key players in the definition of an exon (*Blencowe*, 2000).

Cis acting auxiliary elements are highly variable in sequence but are extremely important in the exon/intron definition. These sequences occur within both exonic and intronic regions and can either promote recruitment of the Spliceosome and exon inclusion (Splicing Enhancers) or disrupt assembly of the Splicing machinery and cause exon skipping (Splicing Silencers). They are normally named according to their location and activity as Exonic Splicing Enhancers (ESEs) and Exonic Splicing Silencers (ESSs), when located in an exon; and Intronic Splicing Enhancers (ISEs) and Intronic Splicing Silencers (ISSs), when located in an intron, as can be seen in Figure 14 (reviewed in *Matlin et al.*, 2005).

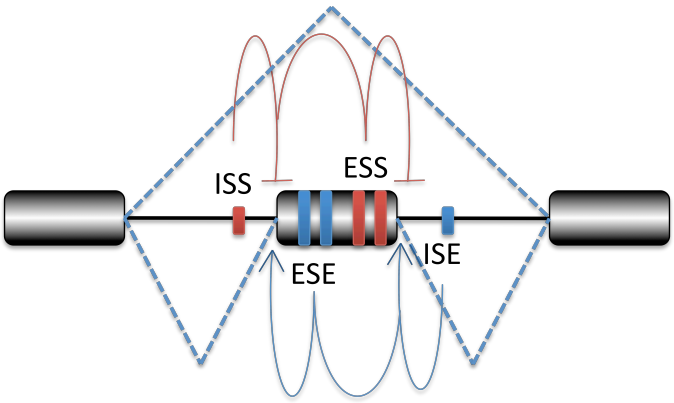


Figure 14 – Splicing auxiliary elements. In addition to the splice-site consensus sequences, a number of auxiliary elements can influence Alternative Splicing. These include Exonic Splicing Enhancers and Silencers (*ESE* and *ESS*, respectively), and Intronic Splicing Enhancers and Silencers (*ISE* and *ISS*, respectively). Enhancers can activate adjacent splice sites or antagonize Silencers, whereas Silencers can repress splice sites or antagonize Enhancers. Silencers are represented in red and Enhancers in blue. Adapted from (*Matlin et al.*, 2005).

While a few regulatory sequences have been shown to function by directly creating RNA secondary structures that alter splice site recognition (*Buratti et al.*, 2004; *Lim et al.*, 2004), the majority act primarily as platforms for binding of non-snRNP regulatory proteins (reviewed in *Matlin et al.*, 2005). Although the binding of each individual protein to *cis* elements on the RNA is weak, a high specificity is achieved through interactions of multiple RNA elements with proteins that can interact with other RNA-binding proteins (*House et al.*, 2007). As a result, exons are recognized with a remarkable fidelity through combinatorial control of different proteins (*Smith et al.*, 2000). Probably the best characterized of the regulatory elements, ESEs, bind a family of proteins known as SR proteins. In contrast, ESS typically function to repress exon inclusion by recruiting members of the hnRNP family of proteins (reviewed in *Stamm*, 2002; *Shin et al.*, 2004; *House et al.*, 2007).

1.2.1.1.3 - The SR and hnRNP families of Splicing regulators

The two more prominent families of non-snRNP Splicing factors consist of serine - arginine (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs).

The SR proteins constitute perhaps the best-studied family of Splicing regulators. They are named SR due to their high Serine (S) and Arginine (R) contents. Members of this family of proteins have multiple functions in the pre-mRNA Splicing reaction. All SR proteins have a modular organization and contain an N-terminal RNA-binding domain (RRM) that interacts with the pre-mRNA and a C-terminal RS domain that functions as a protein interaction domain, as can be seen in Figure 15. The modular structure of the SR proteins, allows that different modules can be exchanged among different family members maintaining their activity. For example, the RNA-binding domains can be exchanged between SR proteins (*Chandler et al.*, 1997; *Mayeda et al.*, 1999) and still bind the RNA in the absence of the RS domain (*Caceres et al.*, 1993; *Zuo et al.*, 1993; *Mayeda et al.*, 1999). The RS domains can also be exchanged among different SR proteins (*Chandler et al.*, 1997; *Wang et al.*, 1998b) and was also found to be possible to function when fused to hnRNP RNA binding proteins (*Graveley et al.*, 1998).

Figure 15 - Schematic diagram of human SR proteins. The domain structures of some of the human SR protein family members. RRM: RNA recognition motif; RRMH: RRM homology and RS: arginine/serine-rich domain. Adapted from (*Graveley*, 2000).

In the cell, SR proteins migrate from speckles - subnuclear domains that may function as storage sites for certain Splicing factors - to sites of active transcription (*Misteli et al.*, 1997; *Misteli et al.*, 1998b; *Misteli et al.*, 1999) and some SR proteins have been found to shuttle in and out of the nucleus (*Caceres et al.*, 1998). The subcellular localization of SR proteins can be modulated by their phosphorylation state (*Misteli et al.*, 1998a; *Misteli et al.*, 1998b). A number of studies have shown that the serine residues within the RS domains of SR proteins are extensively phosphorylated (*Gui et al.*, 1994a; *Misteli et al.*, 1998a). The phosphorylation state of these proteins is involved not only in their distribution within the cell but also in the interactions among the different SR proteins (*Gui et al.*, 1994a; *Wang et al.*, 1998a; *Xiao et al.*, 1998), both of which may change the ability of SR proteins to function in Splicing. It has been reported that both hyper- and hypo- phosphorylated SR proteins are unable to support Splicing (*Prasad et al.*, 1999; *Sanford et al.*, 1999), showing that the SR proteins activity is tightly regulated by their phosphorylation state (reviewed in *Fluhr*, 2008). Several kinases have been identified that can phosphorylate RS domains in vitro. These include the SR protein kinase family (SRPK1 and SRPK2), that only phosphorylates SR proteins (*Gui et al.*, 1994b; *Wang et al.*, 1998a), the dual specificity kinase Clk/Sty family (*Colwill et al.*, 1996) and the DNA topoisomerase I (*Rossi et al.*, 1996; *Labourier et al.*, 1998). Evidence for a role of phosphorylation in alternative splice-site selection has come from the observation that overexpression of Clk/Sty kinase results in a shift of selected splice sites of cellular Clk1 and adenovirus pre-mRNAs *in vivo* (*Duncan et al.*, 1997) and that dephosphorylation induces a splice-site switch in late adenoviral infection (*Kanopka et al.*, 1998).

Mechanisms of Splicing repression are normally associated with the hnRNP family of Splicing factors. In a simplistic way, hnRNP proteins directly antagonize the SR proteins function. HnRNP family is composed of more than 20 proteins that associate with high-molecular-weight nuclear RNA. The members of this family contain RRM-type and KH-type RNA binding domains (*Krecic et al.*, 1999; *Valverde et al.*, 2008), as well as auxiliary domains that are often involved in protein-protein interactions. Many hnRNPs are abundant nuclear proteins that shuttle between the nucleus and cytoplasm (*Dreyfuss et al.*, 1993; *Nakielny et al.*, 1997). hnRNPs interact avidly with themselves and other factors forming the so called hnRNP complexes (reviewed in *Krecic et al.*, 1999). Besides the function of hnRNPs in pre-mRNA Splicing, other functions have been attributed to this family of Splicing factors, such as transcriptional regulation (*Michelotti et al.*, 1996; *Du et al.*, 1998), telomere length maintenance and biogenesis (*Ishikawa et al.*, 1993; *LaBranche et al.*, 1998), polyadenylation (*Bagga et al.*, 1998) and translation control (*Ostareck et al.*, 1997; *Collier et al.*, 1998). As the SR proteins, hnRNP proteins can also be modified post-translationally. Among the best-described modifications is phosphorylation (*Mayrand et al.*, 1993; *Xie et al.*, 2003) and methylation (*Bedford et al.*, 2005). Other post-translational modifications have been reported, although less extensively, such as SUMOylation (*Vassileva et al.*, 2004) and acetylation (*Kim et al.*, 2006).

While much remains to be understood with respect to the mechanism by which these post-translational modifications of SR, hnRNP and other Splicing proteins change in response to extracellular stimuli and the influence in specific Alternative Splicing patterns, many studies already correlated changes in the phosphorylation states of these proteins with signal-induced regulation of several Alternative Splicing events (reviewed in *Black, 2003*).

1.2.2 - Alternative Splicing

"*…from the very limited experimental data at present available to us, is that a chromosomal gene only produces a single protein, whereas a stretch of DNA in virus may produce more than one protein, depending on* ***which way the primary transcript is spliced****...*"

"…in most cases this is because viruses are short of DNA and, by various devices, their limited amount of DNA is made to code for more proteins than would otherwise be possible."

Francis Crick, 1979

As *Francis Crick* observed in 1979 (*Crick*, 1979), several genes show different patterns of Splicing, therefore coding for more than one protein. This was probably one of the first observations of AS and of its importance in the generation of proteomic diversity. Interestingly, an analogy can be made with the Human Genome Project, where it was found that fewer genes exist than it was initially predicted (*Lander et al.*, 2001; *Venter*, 2001), and the necessity of protein diversity generator mechanisms in the cell, where AS plays a major role (reviewed *Black*, 2000; *Maniatis et al.*, 2002; *Kazan*, 2003; *Blencowe*, 2006). However this cannot be attributed exclusively to AS, the use of multiple transcription sites and various post-translational modifications also play a role in the production of proteome complexity (reviewed in *Quelle et al.*, 1995; *Banks et al.*, 2000)

Probably the first report of a gene that undergoes AS dates from 1980, when *David Baltimore* and colleagues findings showed the generation of distinct isoforms, secreted and membrane bound, of the immunoglobulin gene (*Alt et al.*, 1980). If for a long time it was thought that this mechanism only occurred in

exceptional cases, the recent estimates indicate that the expression of approximately 93% of the Human multi-exon genes involves AS (*Pan et al.*, 2008; *Wang et al.*, 2008; *Wahl et al.*, 2009). A typical multiexon pre-mRNA, can undergo multiple Splicing patterns, generating multiple mRNA isoforms of a single gene, therefore generating diversity. There are at least 5 distinct Alternative Splicing patterns described (Figure 16): inclusion or skipping of a cassette exon, selection of an alternative 5' splice site, selection of an alternative 3' splice site, inclusion of only one of two mutually exclusive cassette exons and intron retention. Additional layers of complexity can be added when different 5' and 3' ends of mRNA are generated by the use of alternative promoters and alternative polyadenylation signals present in different exons (*Cartegni et al.*, 2002; *Black*, 2003). These complex combinations of exons and alternative segments show consequently different protein coding sequences.

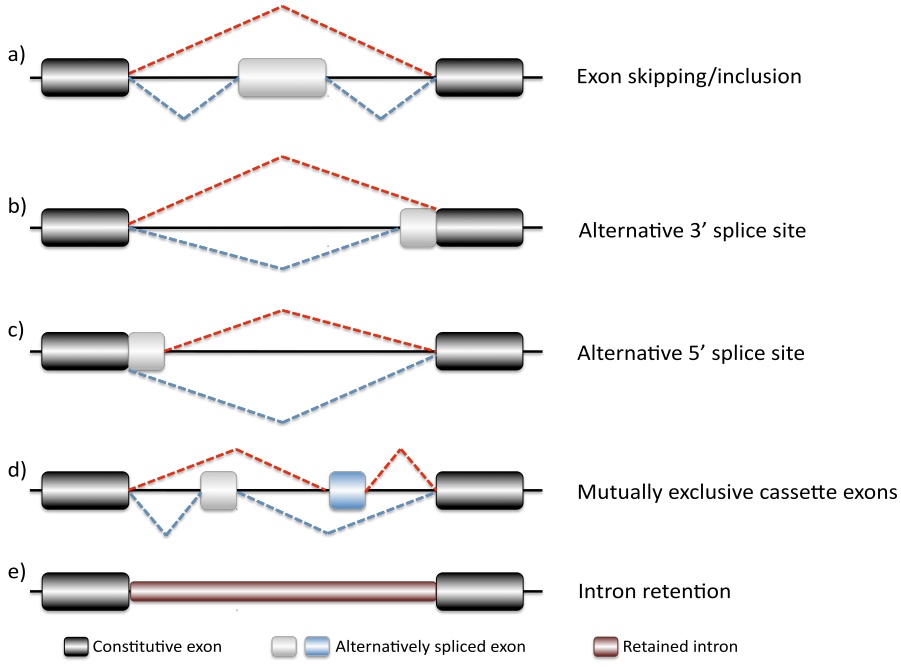


Figure 16 – Patterns of Alternative Splicing. Constitutive sequences present in all final mRNAs are depicted as black boxes. Alternative RNA segments that can either be included or excluded from the final mRNA are represented by light grey and light blue boxes. Retained introns represented as burgundy boxes. a) a cassette exon can either be included or excluded in the mRNA. b) and c) alternative 5' and 3' splice sites allow lengthening or shortening of particular exons. d) two adjacent cassette exons can be included or excluded from the mRNA in a mutually exclusive manner. e) an intron can be retained in the final mRNA. Adapted from (*Cartegni et al.*, 2002).

Besides the major role AS plays as proteome diversity generator, its importance as a regulatory process that contributes to biological complexity through other mechanisms rather than just increasing proteomic complexity has becoming more evident (reviewed in *Lareau et al.*, 2004). Alternative Splicing can regulate gene expression at different levels. A common case occurs when specific domains required for the activity of a certain gene are altered, generating non-functional isoforms, therefore compromising specific gene functions (reviewed in *Vilardell et al.*, 2000; *Lareau et al.*, 2004; *Resch et al.*, 2004;). Another mechanism of gene expression regulation is achieved when AS leads to the production of mRNA isoforms containing premature termination codons, targeting the transcripts for degradation via nonsense-mediated mRNA decay, therefore not available for translation (reviewed in *Lewis et al.*, 2003; *Hillman et al.*, 2004; *Lejeune et al.*, 2005). In addition, the production of specific spliced isoforms where protein-coding regions are not changed may also regulate protein functions . As an example, the generation of different isoforms where the removal or insertion of 3' untranslated region (UTR) regulatory elements can alter mRNA subcellular localization, stability and translation efficiency (reviewed in *Wickens et al.*, 2002; *Bratu et al.*, 2003; *Lareau et al.*, 2004). Alternative mRNAs differing in the 5' UTR are also common and their expression may be regulated through alternative promoter usage (*Zhang et al.*, 2004).

The increasing number of AS related studies have unveilled a great diversity of biological processes where this mechanism plays an important role

such as sex determination, programmed cell death and cell excitation and contraction (reviewed in *Black*, 2003; *House et al.*, 2007). A classical example of AS regulated cellular process, is the regulation of apoptosis (*Jiang et al.*, 1999; *Paronetto et al.*, 2007). The antiapoptotic Bcl-2 family proteins Bcl-2 and Bcl-xL play important roles in inhibiting mitochondria-dependent extrinsic and intrinsic cell death pathways. Alternative Splicing of the Bcl-x gene originates two isoforms, a long (Bcl-xl) and a shorter (Bcl-xs) isoforms with opposite functions, anti- and pro-apoptotic, respectively (*Boise et al.*, 1993; *Youle et al.*, 2008). The mechanism of Bcl-x Splicing has been extensively studied and several Splicing factors (SF) have been implicated in the regulation of Bcl-x Splicing. *Paronetto et al*. (*Paronetto et al.*, 2007) have shown that by modulating the concentration of several SFs, such as Sam68 and hnRNPA1, or by inducing mutations of "key" aminoacid residues, they would induce changes in the Splicing balance of Bcl-x from an non-apoptotic to an apoptotic isoform, thus showing the importance of a tight regulation of this process. As mentioned before in Chapter 1.1.3, a large number of Immune relevant genes, either in the Adaptative or Innate Immune systems, have also been shown to be regulated by AS (reviewed in *Lynch*, 2004; *Wells et al.*, 2006; *Leeman et al.*, 2008).

As a major regulator of different cellular functions, the tight regulation of AS modulates the physiological requirements of a cell. If the control of Alternative Splicing is disrupted, the result can be a failure to meet cellular and tissue requirements resulting in dysfunction and disease. In the past years several diseases have been associated with defects in RNA Splicing (reviewed in *Tazi et al.*, 2008; *Cooper et al.*, 2009). Where defects in general Splicing machinery are usually not compatible with life, changes in Alternative Splicing machinery can be normally tolerated by the organism, but sometimes being a cause of a disease. Although the majority of disease causing mutations has been observed either in *cis* and *trans* Splicing regulatory elements, mutations in core elements of the Splicing

machinery have also been reported. For example, an autosomal dominant form of retinitis pigmentosa is caused by a mutation in the Splicing factors PRPF31/U4-61k and PRP8 (*Vithana et al.*, 2001; *Boon et al.*, 2007; *Wilkie et al.*, 2008). Several diseases have been reported to be caused by point mutations in regulatory sequences (e.g. Spinal muscular atrophy, *Zhang et al.*, 2008), changes in the ratio of protein isoforms generated by AS (e.g. Tauopathies, *Andreadis*, 2005; *Andreadis*, 2006), mutations leading to activation of cryptic splice sites (e.g. Hutchinson-Gilford proegeria syndrome, *De Sandre-Giovannoli et al.*, 2006), mutations in the 5'splice site (e.g. Thalassemias, *Birgens et al.*, 2007). Among the different pathologies where Splicing is playing a role, a recent report by *Xiong et al.* (*Xiong et al.*, 2006) shows that in patients with inflammed muscle, the expression of the Splicing Factor ASF/SF2 is downregulated. Moreover, it was shown that this downregulation can be induced by a pro-inflammatory stimuli, such as TNF-a (*Xiong et al.*, 2006; *Balkwill*, 2009). Therefore, the increasing connection between alterations in AS patterns and disease, and the increased number of cellular processes regulated by AS, strongly suggests that the importance of a tight regulation of this process in different physiological conditions.

We have decided to study the impact of AS regulation in the context of an inflammatory response, namely the secretion of IL-1β after a bacterial challenge. Our objective was to identify molecules involved in Splicing regulation (SFs) that can be implicated in the regulation of IL-1β secretion and their mechanism of action.

Chapter 2 - The Method and Experimental proceedings

"No amount of experimentation can ever prove me right; a single experiment can prove me wrong."

Albert Einstein

2.1 - The method - RNA Interference

"*Andy introduced siRNAs as these small interfering RNAs, as we call them; the little chunks of RNA that go on and silence genes*"

Craig Mello, Nobel Prize lecture 2006

RNA interference (RNAi) originally referred to the ability of exogenously introduced double-stranded RNA (dsRNA) molecules to trigger silencing of complementary messenger RNA sequences in the nematode *Caenorhabditis elegans* (*Fire et al.*, 1998). RNAi is an evolutionary conserved defense mechanism whereby genes are specifically silenced through degradation of messenger RNAs. It became clear that RNAi is mechanistically related to several other conserved RNA silencing pathways, which are involved in the cellular control of gene expression and in protection of the genome against mobile repetitive DNA sequences, such as retroelements and transposons (reviewed in *Dykxhoorn et al.*, 2005; *Grimm*, 2009; *Moazed*, 2009).

2.1.1 - The mechanism

The mechanism by which dsRNA induces gene silencing involves a two-step process. First, RNAi and related gene-silencing pathways are initiated by the production of small RNAs (20-30 nucleotides, *Elbashir et al.*, 2001a; *Elbashir et al.*, 2001b). Several classes of small regulatory RNA described, among them are short interfering RNAs (siRNAs), microRNAs (miRNAs) and PIWI-interacting RNAs (piRNAs, reviewed in *Tolia et al.*, 2007; *Moazed*, 2009). Short interference (siRNAs) and miRNAS are 21-25 nucleotide molecules, which are generated from longer dsRNA precursors by Dicer, a ribonuclease III (RNase III) enzyme (*Bernstein et al.*, 2001; *Ketting et al.*, 2001). These small RNAs subsequently associate with members of the Argonaute family of proteins, which function as the core components of a diverse set of protein-RNA complexes called RNA-induced silencing complexes (RISCs, reviewed in *Dykxhoorn et al.*, 2003; *Tolia et al.*, 2007; *Hutvagner et al.*, 2008). RISCs use the small RNAs as guides for the sequence-specific silencing of messenger RNAs that contain complimentary sequence through inducing the slicing of the mRNAs or repressing their translation. After slicing, the cleaved target RNA is released, and the RISC is recycled for another round of slicing (Figure 17, reviewed in *Dykxhoorn et al.*, 2003; *Dykxhoorn et al.*, 2005; *Tolia et al.*, 2007).

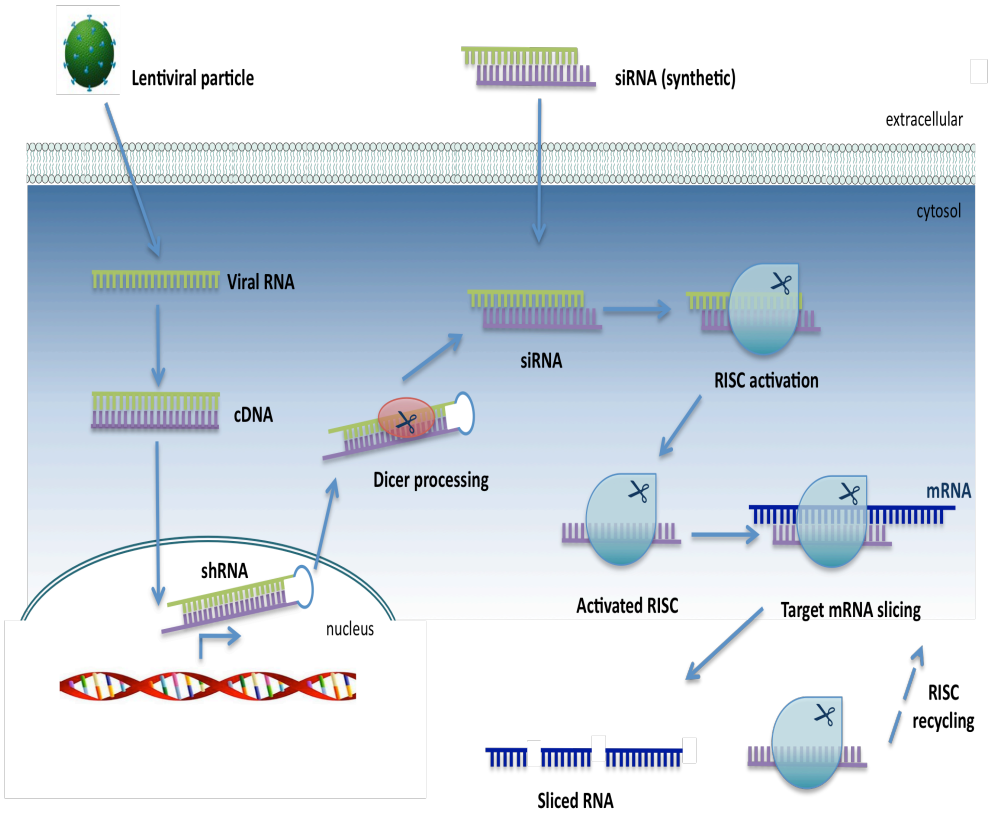


Figure 17 – RNAi interference pathway, shRNA and synthetic siRNA delivered. The effector molecules that guide mRNA degradation are small 21-25 nucleotide dsRNA, termed short interfering RNA (siRNA), produced by the cleavage of long dsRNA. These siRNA are produced by the cytoplasmic Dicer family of RNase III-like enzymes, resulting in a characteristic 21-23 nucleotides dsRNA duplex with 2-3 nucleotide overhangs. The siRNAs are taken up into a multi-subunit ribonucleoprotein complex called RNA-induced silencing complex (RISC). The antisense (guide) strand of the siRNA directs the endonuclease activity of RISC to the homologous (target) site on the mRNA, resulting in mRNA slicing and RISC recycling for posterior rounds of silencing. RNAi can be induced by the introduction of synthetic short interfering RNA (siRNA) or by intracellular generation of siRNA from vector driven expression of the precursor short hairpin RNA (shRNA). In the latter method, upon delivery of the shRNA expression vector into the cytoplasm (e.g. using lentiviruses), the viral RNA is reverse transcribed into complementary DNA (cDNA), by the viral Reverse Transcriptase. cDNA is then integrated into the host cell genome, and posterior transcribed by the RNA polymerase II or III. The primary transcript generated by the RNA polymerase contains a hairpin like stem-loop structure therefore named shRNA. This shRNA is then exported to the cytosol where it follows the natural RNAi pathway, processed by Dicer, loaded on RISC and further promoting complimentary mRNA degradation. Adapted from (*Jinek et al.*, 2009).

2.1.2 - RNAi as a research tool. Screening Era

Since its discovery, RNAi have been extensively used as a tool to understand gene function *in vitro* and *in vivo*. RNAi is an extremely powerful research tool to perform reverse genetic studies, which focus in undermining the function of a certain gene by its disruption. Silencing of target genes can be mediated by introducing synthetic siRNAs or by vector systems (e.g. lentiviral vectors, *Stewart et al.*, 2003)) that encode short hairpin RNAs (shRNA) that are processed intracellular into siRNAs (Figure 17, reviewed in *Dykxhoorn et al.*, 2003; *Manjunath et al.*, 2009; *Rao et al.*, 2009). Typically, RNAi mediated silencing is incomplete, i.e. it is a "knockdown" and not a "knockout", although in some cases, the targeted mRNA is undetectable even with very sensitive PCR assays (*Song et al.*, 2003; *Rao et al.*, 2009). However, the incomplete silencing performed by RNAi might be useful in certain studies, where the complete silencing of a gene would compromise the survival of the cell/organism and therefore not possible to knockout (e.g.ASF/SF2, *Cooper*, 2005). The progress in genome sequencing and annotation, as well technological advances that allow biological assays to be executed and analyzed in high throughput manner, triggered an increasing interest in genome-wide RNAi based screens, where the effects of gene silencing on biological phenotypes can be systematically explored. This approach has been pursued most extensively in simpler organisms, such as *C.elegans*, and more recently in *Drosophila* and human cells (reviewed in *Fraser et al.*, 2000; *Berns et al.*, 2004; *Boutros et al.*, 2004; *Kittler et al.*, 2004; *Kolfschoten et al.*, 2005; *Pelkmans et al.*, 2005; *Westbrook et al.*, 2005; *Friedman et al.*, 2007; *Oberdoerffer et al.*, 2008). Cell-based RNAi screens can be performed either using arrays of individual RNAi constructs in high-throughput screening protocols, or by using pools of different RNAi constructs, termed

arrayed or pooled screens, respectively (*Moffat et al.*, 2006; *Sharma et al.*, 2009). Pooled screens have the advantage that a large set of RNAi molecules, perhaps targeting an entire genome, can be delivered to bulk populations of cells, thereby allowing rapid evaluation of large gene sets and/or parallel analyses of multiple cell types. This type of screen is typically less expensive and more practical to do in a standard lab space, whereas arrayed screening normally requires specialized high-throughput equipment and is more expensive. However, the deconvolution process of the putative "hits" can be really laborious, being a disadvantage of the pooled screen. In other hand, in arrayed screens, each unique RNAi construct (targeting a single gene) is delivered to a cell subset, therefore allowing the rapid identification of the gene responsible for the phenotype observed, with a lower extent of false negatives when compared to the pooled screens (*Root et al.*, 2006).

Several libraries to perform gene silencing in a different number of organisms, such as mouse and human, have already been built and used successfully (*Berns et al.*, 2004; *Kittler et al.*, 2004; *Paddison et al.*, 2004; *Silva et al.*, 2005; *Moffat et al.*, 2006). Among those libraries, is the The RNAi Consortium (TRC) lentiviral library that covers the entire human and mouse genomes, with multiple constructs targeting each gene (reviewed in *Moffat et al.*, 2006; *Root et al.*, 2006).

2.1.2.1 - TRC lentiviral library

The TRC library was designed to cover entire human and mouse genome, with a five-fold coverage, meaning that has an average of five shRNA constructs to target each gene (reviewed in *Moffat et al.*, 2006; *Root et al.*, 2006). This library allows high-efficiency delivery of the shRNA and enables stable long-term gene

suppression in a broad range of cell types, including primary cells (*Boehm et al.*, 2007; *Oberdoerffer et al.*, 2008; *Savina et al.*, 2009), cell lines (*Boehm et al.*, 2007), dividing and nondividing cells (*Moffat et al.*, 2006). Briefly, the library was cloned into a lentiviral vector, pLKO.1, which drives shRNA expression from a human U6 promoter and carries the puromycin-resistance gene, allowing the selection of transduced cells (Figure 18). Like other third-generation lentiviral vectors (*Dull et al.*, 1998), viruses are generated using a three-plasmid packaging system, separating the HIV *gag*, *pol* and *rev* genes, and the gene encoding the Vesicular stomatitis virus (VSV)-G coat onto separate vectors, therefore producing self-inactivating viruses, minimizing the possibility of recombination to create replication-competent viruses (*Moffat et al.*, 2006; *Root et al.*, 2006). Upon infection of the target cell, viral genome is integrated into the host cell genome. After integration, the cassette produces an shRNA molecule that will be processed by the RNAi machinery and target the degradation of the complimentary mRNA, as described above (Figure 17).

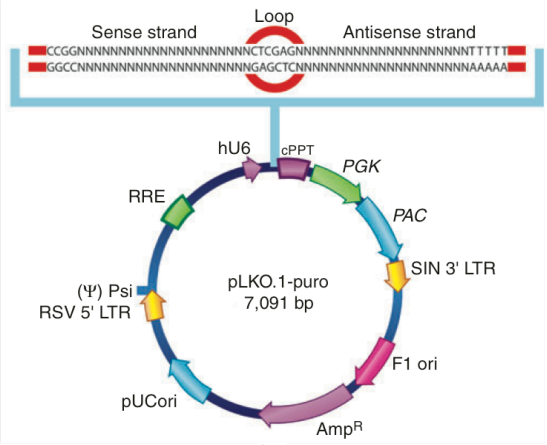


Figure 18 – Map of the TRC lentiviral vector pLKO.1. Expression of the shRNA cassette is driven by the human U6 RNA polymerase II promoter (hU6). The lentiviral vector also contains the mammalian selection marker puromycin resistance gene (PAC) under the control of the PGK promoter, allowing the selection of transduced cells. Ampicilin resistance gene (Amp^R) allows for colony selection upon bacterial transformation with the lentiviral vector. Adapted from (*Root et al.*, 2006).

2.1.2.1.1 - TRC library subsets

RNAi based screenings can be performed in a genome-wide fashion, targeting the organism entire genome, or in a more accessible manner, by targeting specific gene subsets, such as kinases, phosphatases, NLRs, Splicing factors, among others. A Splicing related subset was reorganized from the main TRC library, encoding all known Splicing factors, Splicing regulators and proteins shown to interact with Spliceosome components, according to was known in the literature (*Grabowski et al.*, 1985; *Krecic et al.*, 1999; *Sanford et al.*, 1999; *Longman et al.*, 2000; *Makarov et al.*, 2002; *Maniatis et al.*, 2002; *Zhou et al.*, 2002; *Barbosa-Morais*, 2005; *Wang et al.*, 2006). A similar approach was performed to compile the Caspases, CARD containing proteins and NLRs shRNA collections (*Earnshaw et al.*, 1999; *Elkon*, 1999; *Martinon*, 2004; *Inohara et al.*, 2005; *Kufer et al.*, 2005; *Martinon et al.*, 2005; *Ting et al.*, 2005; *Kersse et al.*, 2007; *Mariathasan et al.*, 2007; *Martinon et al.*, 2007b; *Park et al.*, 2007). The complete lists of the genes that compose each collection are shown in chapter VIII-1 and VIII-2 (Tables S1 and S2).

2.1.2.1.2 - RNAi based screening outline

THP-1 cells were infected with the shRNA-containing vector, as described in the lentiviral infection procedure (chapter II-2.4). After the 3 days of puromycin selection, plates were divided into 2. In one of the plates cell number was assayed by Alamar Blue® cell viability assay (Invitrogen®), according to manufacturer's protocol. On the other plate, cells were stimulated with 4% PFA fixed DH5α *E.coli* at a Multiplicity of Infection (MOI) of 20 bacterial cells per THP-1 cell. Twenty-four hours after stimulation, cell supernatants were collected and submitted to

ELISA assay for the quantification of IL-1β and IL-8 cytokines, according to ELISA protocol present at chapter II-2.5. Cytokine concentration was then calculated per cell number. A schematic view of the RNAi based screening outline can be seen in Figure 19.

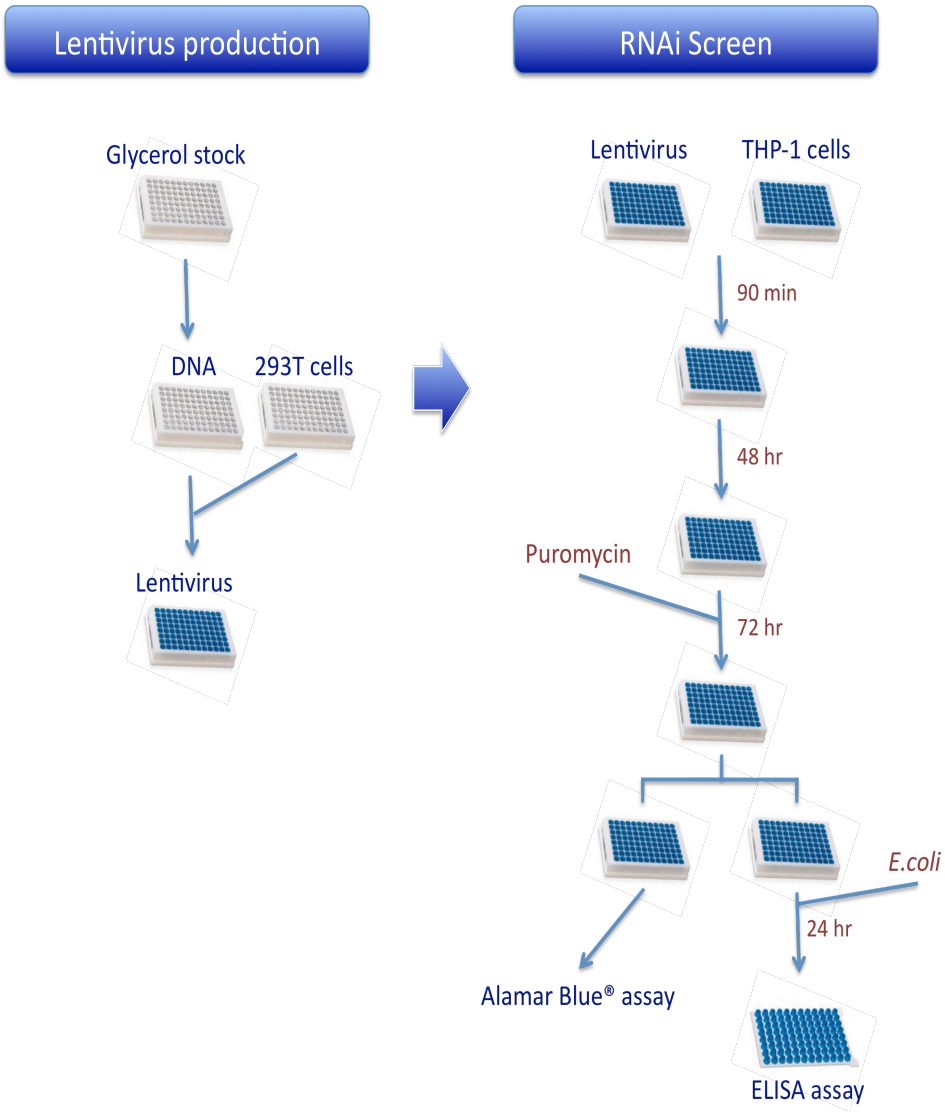


Figure 19 – Lentiviral production and RNAi based screening outline. TRC library is kept in glycerol stocks at -80ºC. Bacterial stocks transformed with the pLKO.1 plasmids were incubated at 37ºC and plasmid was purified by a high-throughput plasmid purification protocol (as described in chapter 4.2). 293T cells were transfected with library pLKO.1 and the packaging plasmids to produce the lentiviral vectors (as described in chapter 4.3). Lentiviruses were used to transduce THP-1 cell line. Briefly, lentiviral infection was performed by 90 minutes spinoculation (2200 RPM at 37ºC) of THP-1 cells with the lentiviral vectors. After spinoculation, media was replenished and cells incubated for 48 hr. In order to select the transduced cells, puromycin was added and cells incubated for 72 hr. After puromycin selection, each 96 well plate was divided into 2 to be able to perform an Alamar Blue ® cell viability assay and to stimulate the remaining plate with 4%PFA fixed *E.coli*. After 24 hr *E.coli* stimulation, cell supernatants were collected and assayed for cytokine concentration by ELISA (as described in chapter II.2.5).

2.2 - Experimental proceedings

2.2.1 - Cell culture

THP-1 cells (ATCC TIB-202) were grown in R10- RPMI media 1640 (GIBCO ®) supplemented with 10% (v/v) Fetal Bovine Serum (GIBCO ®), 1% (v/v) Penicillin-Streptomycin (GIBCO ®), 1% (v/v) Pyruvate (GIBCO ®), 1% (v/v) L-Glutamine (GIBCO ®), 1% (v/v) Non-essential aminoacids (GIBCO ®), 1% (v/v) Hepes buffer (GIBCO ®) and 2-Mercaptoetanol (GIBCO ®) to a final concentration of 0.05M, as recommended by the American Tissue Culture Collection (ATCC). HEK 293T cells (ATCC CRL-11268) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO ®) supplemented with 10% (v/v) Fetal Bovine Serum (GIBCO ®), 1% (v/v) Penicillin-Streptomycin (GIBCO ®) and 2-Mercaptoetanol (GIBCO ®) to a final concentration of 0.05M. Cells were kept at 37ºC under a 5% carbon dioxide (CO_2) atmosphere.

2.2.2 - hairpin-pLKO.1 Plasmid preparation

Plasmids were prepared according to TRC proceedings listed at (https://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). 96 deep well sterile growth plates (Corning ®) were pre-filled with 1.2 mL of Terrific broth (TB, Invitrogen ®) media containing 100 ug/mL Carbenicillin (Sigma-Aldrich®). The hairpin-pLKO.1 containing bacterial glycerol stocks were inoculated on the TB pre-filled 96 deep well plates and grown for at least 16 hours at 37ºC under agitation, 300 rpm. After incubation, plates were centrifuged for at 1500 g for 8 minutes at 4ºC, and supernatants discarded. Bacterial cell pellets were ressuspended in 200 uL of RNase A containing Ressuspension buffer (50 mM Tris-HCl, 10 mM EDTA pH 8.0 and 0.1 mg/mL RNase A) and further lysed in 210

uL fresh Alkaline Protease containing lysis buffer (200 mM NaOH, 1% (w/v) SDS and 25 units/mL of Alkaline Protease) for 4 minutes at room temperature. Bacterial cell lysates were neutralized by adding 300 uL Neutralization buffer (3.75 M Guanidinium Hydrochloride, 0.9 M KOAc and 1.4 M HOAc, pH4.35). After 30 minutes 4ºC centrifugation at 3000g, lysates were transferred to a clarification filter plate (Whatman®) and centrifuged at 3000g for 5 minutes at 4ºC. Clarified lysates were then incubated at 70ºC for 30 minutes. After incubation, lysates were transferred to a pDNA binding plate (Whatman®), centrifuged at 1800g for 2 minutes at 4ºC and supernatants were discarded. Two washes were performed by centrifuging the pDNA binding plates at 1800g for 2 minutes at 4ºC in the presence of 600 uL of Wash buffer (480 mL of 100% ethanol added to a 120 mL of a solution containing 15 mM NaCl, 40 mM Tris-HCl and 25 mM Tris, pH 6.65). Plasmid DNA was eluted from the pDNA binding plates by centrifuging at 1800g for 5 minutes at 4ºC after an incubation of 10 minutes with 140 uL of Elution buffer (10 mM Tris-HCl, pH 8.0). Plasmid DNA concentration was assayed by spectrophotometry in a Tecan Infinite® 200 equipment using Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen®) following manufacturer's protocol, and further stored at -80ºC.

2.2.3 - Lentivirus production

Lentiviruses were produced according to TRC lentiviral proceedings present at the RNAi Consortium website (https://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). Briefly, HEK 293T cells were seeded at a density of $2.2x10^5$ cells/mL (100 uL per well in 96 well plates) in DMEM media without Penicillin-Streptomycin. After 24 hr

incubation at cell culture normal conditions, cells were transfected with a mixture of the 3 plasmids, the packaging plasmid (100 ng of pCMV-dR8.91), the envelope plasmid (10 ng of VSV-G/pMD2.G) and 100 ng of the shRNA containing pLKO.1-puro vector, using TransIT-LT1 transfection reagent (Mirus Bio ®) in Optimem medium (GIBCO ®), following TRC transfection proceedings. After 18 hours of incubation, media was replaced with 170 uL of high serum growth media (30% FBS (v/v) DMEM). Twenty-four hours after media replacement, viruses were harvested (150 uL) and fresh media (150 uL) was added to the cells. The second viral collection was performed 24 hours later and harvested viruses were pooled with the previous day collection and stored at -80ºC.

2.2.4 - Lentiviral infection

Lentiviral infection was performed according what is described at the RNAi Consortium website (https://www.broadinstitute.org/genome_bio/trc/publicProtocols.html). Briefly, 10 uL of viruses were plated per well in a 96 well plate. THP-1 cells were ressuspended in R10 medium containing 8 ug/mL of Polybrene (Sigma-Aldrich ®) at a density of $1.25x10^6$ cells/mL, and 40 uL were added on top of the virus containing wells. Plates were spun for 90 minutes at 2200 RPM at 37ºC. After spinoculation, media was removed and replaced with 200 uL of fresh R10, and cells incubated at 37ºC under normal cell culture conditions. After 2 days, transduced cell selection was performed by adding 50 uL of R10 containing Puromycin (Calbiochem®) to a final concentration of 5 ug/mL. Cells were kept under Puromycin selection for 3 days and then were ready to be used in further assays.

2.2.5 - ELISA Assay

Cytokine concentration (IL-1β and IL-8) in cell supernatants were assayed by Enzyme-linked immunosorbent assay (ELISA) using Human IL-1 beta/IL-1F2 DuoSet® and Human CXCL8/IL-8 DuoSet® (R&D Systems ®), respectively, according to company's protocol. Briefly, 96 well plates (Nunc®) were coated with 50 uL of 4 ug/mL capture antibody diluted in PBS and incubated overnight at 4ºC. Plates were washed 3 times with 0.1% PBS-T previous to 1 hour room temperature blocking step with 100 uL of I-Block™ Protein-Based Blocking Reagent (Applied Biosystems®). After blocking, plates were washed 3 times with 0.1% PBS-T and 50 uL of samples were added to each well, including the respective standard controls at different concentrations ([IL-1β]=0-250 pg/mL and [IL-8]=0-2 ng/mL), and incubated for 1 hour at room temperature. Three more washes were performed with 0.1% PBS-T and 50 uL of respective detection antibodies diluted in I-Block™ Protein-Based Blocking Reagent (100 ng/mL and 20 ng/mL, for IL-1β and IL-8 detection, respectively) were applied to each well and plates were incubated for 1 hr at room temperature. Fifty microlitres of Streptavidin (R&D Systems ®) diluted in PBS (1:200 (v/v)) was added to the plates after the 3 washes with 0.1% PBS-T. After 30 minutes of incubation with streptavidin, plates were washed for another 3 times with 0.1% PBS-T, developed with of 50 uL of Pierce® Soluble TMB Substrate. The developing reaction was stopped by adding 50 uL of 2 N H_2SO_4. Plates were read at 450 nM using a Tecan Infinite® 200 equipment.

2.2.6 - Quantitative RT-PCR

Total RNA was extracted using TRIzol® reagent (Invitrogen®) and final RNA quality and concentration determined by spectrophotometry using Nanodrop 2000c equipment (Thermo Scientific®). Complementary DNA (cDNA) synthesis was carried out using Superscript II Reverse Transcriptase (Invitrogen®) according to manufacturer's directions. Quantitative RT-PCR (qRT-PCR) was performed in the presence of Power SYBR® green PCR Master Mix (Applied Biosystems®) and the amplification protocol was performed on a Rotor-Gene 6000 (Corbett®). All samples were normalized against the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and the relative expression of each gene was calculated using *Pfaffl*'s method (*Pfaffl*, 2001). The primers were designed using different online softwares, namely Primer3 (http://frodo.wi.mit.edu/primer3/), MGH/Harvard Primer bank (http://pga.mgh.harvard.edu/primerbank/) and Primer-blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The complete list of primer sequences is shown in Table S5.

2.2.7 - Western-blot

THP-1 cells from the different experiments were collected and washed twice with cold Phosphate Buffered Saline (PBS) solution. Cells were lysed for 15 minutes at 4ºC using RIPA buffer (50 mM Tris-HCl at pH=7.4, 1% NP-40, 0.25% Sodium Deoxicholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1 mM NaF in the presence of proteases inhibitor cocktail (Roche®)). Lysed cells were centrifuged at 14000 RPM for 15 minutes at 4ºC, and supernatants kept at -20ºC.

Cell lysates were denatured at 95ºC for 10 minutes in the presence of Laemli buffer (Biorad®) before loaded on 10% / 12% Sodium Dodecyl Sulfate (SDS)-PAGE gels. Electrophoresis was performed at 110 V for 2 hours, and gels transferred to nitrocellulose membranes (Whatman®) at 220 mA for 1 hour. Membranes were blocked in a solution containing 5% Milk for one hour and incubated with primary antibodies for one hour at room temperature, just for β-actin antibody, or overnight at 4ºC, for all the other antibodies (complete list of antibodies and dilutions used, in Table S3). Before applying the secondary antibody, membranes were washed 3 times with 0.1% PBS-T (PBS plus 0.1% Tween20 (Sigma-Aldrich®)). Secondary antibody incubations were performed for 1 hour at room temperature, followed by 3 times 10 minutes washes with PBS-T, before developing with ECL plus reagent (GE Healthcare®). Membranes were developed in an AGFA Curix 60® equipment.

2.2.8 - Caspase-1 activity assay by FACS

Caspase-1 activity was measured using the Carboxyfluorescein FLICA Detection kit for Caspase Assay (Immunochemistry Technologies, LLC®) following the reagent instructions. Briefly, cells from the different assays performed were incubated for 1 hour at 37ºC with 30X FLICA solution at a 1:30 ratio, protecting the cells from light exposition. Cells were collected into FACS tubes (BioRad®) and washed 3 times using the provider's wash buffer. The washes were performed by adding 500 uL of wash buffer and tubes centrifuged at 400g for 5 minutes at room temperature. After the final wash, cells were ressuspended in 150 uL of wash buffer and immediately assayed by Flow citometry, using a FACSCalibur system (BD biosciences®). The data collected was further analysed using FlowJo software (Tree Star Inc®).

2.2.9 - Construction of overexpressing lentiviral vectors

Lentiviral vectors for protein overexpression were constructed using the Gateway® pENTR™11 vector and pLenti6/V5-DEST Gateway® Vector kit from Invitrogen®. SFRS1, SFRS3, IL-1β and IL-18 were amplified from THP-1 cells cDNA by PCR reaction using specific primers and annealing temperatures as mentioned at Table 2. All PCR reactions were performed with Phusion® High-Fidelity DNA Polymerase (Finnzymes®) and using the following cycle conditions: initial denaturating step for 5 minutes at 95ºC; 40 cycles (30 seconds at 95ºC, 1 minute at annealing temperature (mentioned on Table 3), extension step for 1 minute at 72ºC and a final elongation step at 72ºC for 7 minutes.

Table 3- Primers used for cloning of SFRS1, SFRS3, IL-1β and IL-18.

Gene	Primers	Annealing (ºC)	Amplicon (bp)
SFRS1	**cloneSFRS1_Fw-** ATAGGATCCTTATGTCGGGAGGTGGT	52.9	766
	cloneSFRS1_Rw- TTGCGATATCTTGTACGAGAGCGAGA		
SFRS3	**cloneSFRS3_Fw-** ATCGGATCCTTATGCATCGTGATTCC	64.1	514
	cloneSFRS3_Rw- GCGCGATATCTTTTCCTTTCATTTGACCT		
IL-1β	**cloneIL-1β_Fw-** ATAGGATCCTTATGGCAGAAGTACCT	52.9	829
	cloneIL-1β_Rw- TATTGATATCTGGAAGACACAAATTG		
IL-18	**cloneIL-18_Fw-** ATAGGATCCTTATGGCTGCTGAACCA	52.9	601
	cloneIL-18_Rw- TGCCGATATCTGTCTTCGTTTTGAAC		

After amplification, PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen®). Purified products and empty pENTR11 vector (Invitrogen®) were digested for 90 minutes at 37ºC using EcoRV and BamHI restriction enzymes (New England Biolabs®). After digestion, pENTR11 vector was dephosphorylated for 30 minutes at 37ºC in the presence of Antartic Phosphatase enzyme (New England Biolabs®). A ligation step was performed using T4 DNA ligase enzyme (New England Biolabs®) for 75 minutes at room temperature, followed by 10 minutes at 65ºC. Ligation products were used to transform DH5α competent bacterial cells (Invitrogen®). Transformed bacteria were inoculated in Lysogeny Broth (LB, Invitrogen®) agar plates in the presence of 50 ug/mL Kanamycin (Calbiochem®) and incubated overnight at 37ºC. Colonies were inoculated in 50 ug/mL Kanamycin containing TB media and incubated overnight at 37ºC under agitation, 300 rpm. Plasmid extraction and purification was performed using PureYield™ Plasmid Miniprep System (Promega®) following manufacter's protocol. Purified plasmids were sequenced using the following primers (**Seq_Fw**-TTTCTACAAACTCTTCCTGTTAGTTAGT and **Seq_Rw**-GTAACATCAGAGATTTTGAGACAC). A map of pENTR11-SFRS1 plasmid can be seen in Figure 20, all the others are in shown in Figure S2.

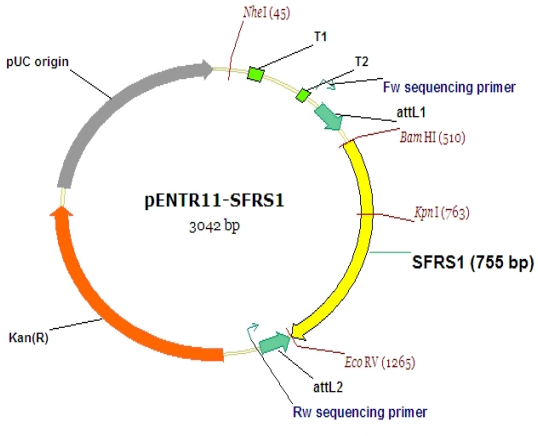


Figure 20 – pENTR11-SFRS1 map. SFRS1 gene was cloned into a BamHI and EcoRV sites in the multiple cloning site of the pENTR11® vector. Two restriction sites can be used to check insert orientation after the ligation step, NheI and KpnI. After transformation into bacterial cells, kanamycin selection can be performed due to a kanamycin resistance gene (Kan(R)) present in the final vector. Sequencing primers were designed in the outside of the multiple cloning site, therefore can be used for all the constructs performed.

pENTR11 vectors were then recombined into a lentiviral vector, the pLenti6/V5-DEST Gateway® Vector (Invitrogen®) following the company's protocol. Briefly, pENTR11 vectors were recombined with pLENTI6/V5-DEST in the presence of Gateway® LR Clonase® II enzyme mix (Invitrogen®) at 25ºC for 150 minutes, followed by 10 minutes of Proteinase K (Roche®) treatment at 37ºC. Stbl3™ competent *E.coli* cells (Invitrogen®) were transformed with the recombined vector, inoculated on LB agar plates in the presence of 100 mg/mL of Ampicillin (Calbiochem®) and incubated for 16 hours at 37ºC. Bacterial colonies were inoculated in 100 ug/mL Ampicillin TB media and incubated for 16 hours at 37ºC. pLENTI6/V5-DEST DNA encoding the gene of interest was purified using PureYield™ Plasmid Miniprep System (Promega®) and concentration measured by spectophotometry in a Nanodrop 2000c equipment (Thermo Scientific®). pLENTI6/V5-DEST-SFRS1 map can be seen in Figure 21, all the others are in shown in Figure S2. Lentiviruses were produced using the same protocol as described for the expression of pLKO.1 vector.

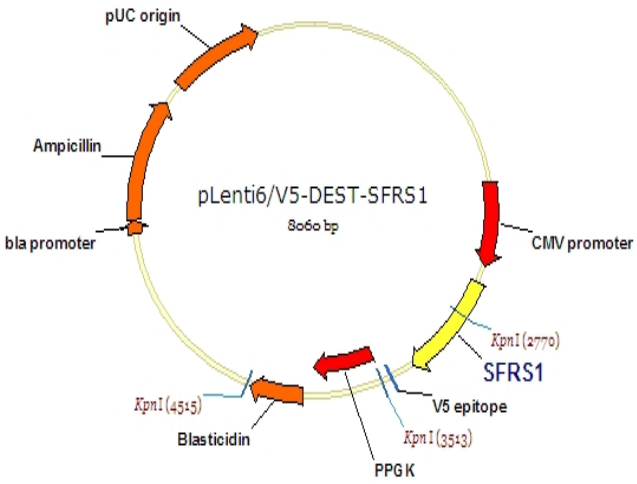


Figure 21 – pLenti6/V5-DEST-SFRS1 map. pENTR11-SFRS1 was recombined into a pLenti6/V5-DEST Gateway® Vector (Invitrogen®). The expression of SFRS1 is driven by the Human cytomegalovirus (CMV) immediate early promoter, which allows high-level and constitutive expression of the gene of interest (*Boshart et al.*, 1985). A PPGK promoter allows high-level expression of the Blasticidin resistance gene, allowing for transduced cells selection (*Kimura et al.*, 1994). Ampicillin resistance gene allows selection of the plasmid in *E. coli*, while pUC origin permits high-copy replication and maintenance in *E.coli* cells. Several restriction sites such as KpnI can be used to digest the plasmid and check for insert orientation and expression. Insert expression in mammalian cells can be visualized by the detection of the V5 epitope.

2.2.10 - Microarray analysis and validation

Microarray data was analyzed in collaboration with *Ana Grosso* (UBCe, IMM) using R software and suitable packages available from CRAN® (*Team*, 2009) and Bioconductor (*Gentleman et al.*, 2004). The raw data for the Affymetrix GeneChip Exon microarray data sets was normalized and summarized using

FIRMA method (*Purdom et al.*, 2008) implemented in aroma.affymetrix package (*Bengtsson et al.*, 2008). The statistical significance from the gene and exon expression alterations was assessed using linear models and empirical Bayes methods (*Smyth*, 2004) implemented in limma package (*Smyth*, 2005). Graphical representation of Firma scores for each probe set or probe selection region (PSR) was based on annotated exons from ExonMine (*Mollet et al.*, 2009).

Chapter 3 - Results

"If it can't be expressed in Figure*s, it is not science, it is opinion."*

Lazarus Long

3.1 - Human monocytic cell line (THP-1) as a model to study regulation of IL-1β secretion

3.1.1 - *E.coli* challenge induces IL-1β secretion

The Human monocytic cell line (THP-1) has been extensively used in a number of studies addressing different Innate Immune responses to several PAMPs, namely the mechanisms of Inflammasome activation and regulation (*Martinon et al.*, 2002; *Agostini et al.*, 2004; *Bruey*, 2004; *Damiano et al.*, 2004). In fact, the Inflammasome discovery was performed using THP-1 cells (*Martinon et al.*, 2002). The ability of THP-1 cells to produce IL-1β was demonstrated by different independent studies, however the kinetics of IL-1β production and processing has not been assessed before in response to PFA-fixed *E.coli*. We stimulated THP-1 cells with 4%PFA-fixed *E.coli* and collected cells and supernatants at different time points after the bacterial challenge. Increased IL-1β mRNA expression was observed after challenge, detected by qRT-PCR analysis (Figure 22-a). Moreover, it is already highly induced at 4 hours after bacterial stimulation.

In order to verify if increased IL-1β mRNA expression is related with augmented cytokine secretion, we quantified the secreted levels of IL-1β in the cell supernatants by ELISA. As can be seen in Figure 22-b, IL-1β secretion is already detected at 2 hours of *E.coli* challenge. The secretion of this cytokine keeps raising even at 24 hours after challenge, peaking at around 48hr post-stimulation (data not shown).

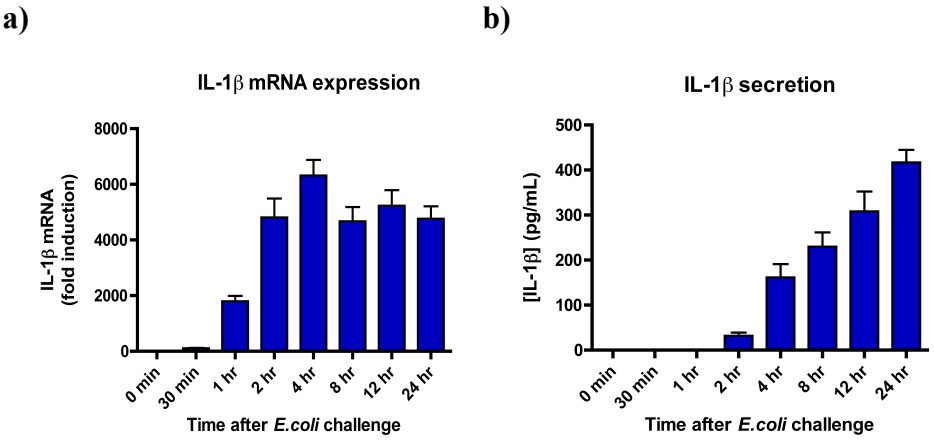


Figure 22 – Kinetics of IL-1β mRNA expression and secretion after *E.coli* stimulation. THP-1 cells were challenged with 4% PFA-fixed *E.coli*. Cells and supernatants were collected at different time points post-stimulation. a) IL-1β mRNA expression was assayed by qRT-PCR. b) IL-1β secretion were assayed in cell supernatants by ELISA. IL-1β mRNA expression and secretion is induced in THP-1 cells by *E.coli* challenge.

In addition to IL-1β, we decided to evaluate the expression of other pro-inflammatory mediator, the Interleukin-8 (IL-8). Interleukin-8 belongs to a chemotactic cytokine family and is responsible for the chemotactic migration of neutrophils and other cell types (such as monocytes) to the sites of Inflammation (reviewed in *Miller et al.*, 1992; *Feghali et al.*, 1997). Interleukin-8 is produced by a wide variety of cell types, however, like IL-1β, monocytes and macrophages typically represent the principal cellular source. Multiple stimuli have been shown to induce the secretion of IL-8, including LPS and live bacteria (*Standiford et al.*, 1990; reviewed in *Remick*, 2005). In opposition to IL-1β, IL-8 is actively secreted into the extracellular space following stimulation of cell with no processing requirements (reviewed in *Remick*, 2005). As can be seen in Figure 23, increased IL-8 mRNA expression and secretion is observed following *E.coli* stimulation of

THP-1 cells. However, in comparison with IL-1β secretion, IL-8 secreted levels peak at a much earlier time point after the bacterial challenge. This can be explained by the requirement of IL-1β to be processed in order to be secreted, whereas IL-8 processing is not needed, therefore peaking earlier (*Martinon et al.*, 2002; *Remick*, 2005; *Dinarello*, 2009).

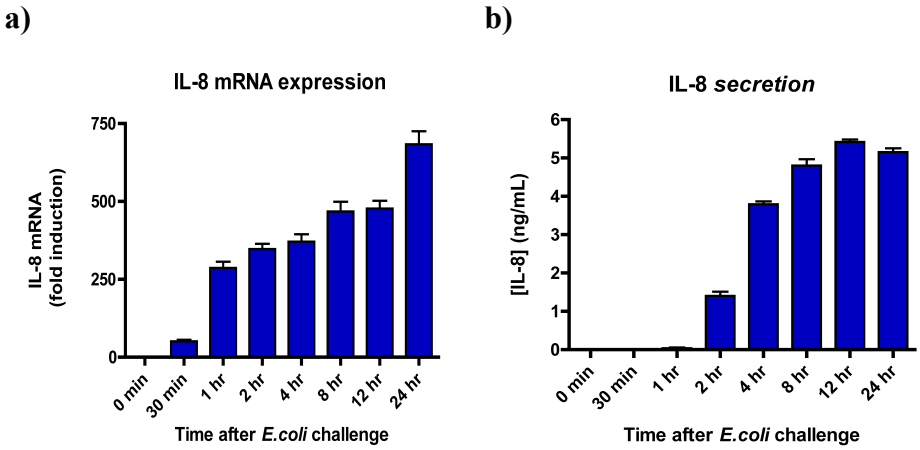


Figure 23 – Kinetics of IL-8 mRNA expression and secretion after *E.coli* stimulation. THP-1 cells were challenged with 4% PFA-fixed *E.coli*. Cells and supernatants were collected at different time points post-stimulation. a) IL-8 mRNA expression was assayed by qRT-PCR. b) IL-8 secreted levels were assayed in cell supernatants by ELISA. IL-8 mRNA expression and secretion is induced in THP-1 cells by *E.coli* challenge.

The expression of both pro-inflammatory cytokines can be detected after challenging THP-1 cells with *E.coli*, therefore we have decided to use this cell line to study IL-1β regulation. We used the 24 hr challenge with *E.coli* as the main time point for the following studies.

3.1.2 - IL-1β secretion is Caspase-1 dependent

As described in Chapter 1.1 (Figure 1), IL-1β release is dependent on the processing of its inactive form (pro-IL-1β) into a mature form. The processing step is mainly mediated by Caspase-1, however several other proteases have been shown to be able to process IL-1β (reviewed in *Netea et al.*, 2010). Therefore we used different strategies, either knocking-down Caspase-1 expression by RNAi or blocking Caspase-1 activation using specific inhibitors, in order to test this dependency on Caspase-1 for IL-1β release following *E.coli* challenge.

As can be seen in Figures 24 (a, b and c), knocking-down Caspase-1 by RNAi almost completely abolished IL-1β secretion after 24 hr *E.coli* challenge. Similar results were obtained when using drugs that inhibit Caspase-1 activity. These drugs have been extensively used to block Caspase-1 activity in different experimental sets using different cell lines (*Margolin et al.*, 1997; *Watson et al.*, 1998; *Gurcel et al.*, 2006; *Keller et al.*, 2008), and were shown to compete with the common substrates of Caspases for the binding to the catalytical site in a reversible or irreversible manner, therefore inhibiting its activity. As can be seen in Figure 24-d, the secretion of IL-1β is reduced in the presence of all inhibitors tested, either using the broad Caspase inhibitors (Z-VAD and Z-DEVD) or a more specific Caspase-1 inhibitor (Z-YVAD). Thus, these data confirms the previous results showing that Caspase-1 and its processing activity are required to process pro-IL-1β into the mature secreted form.

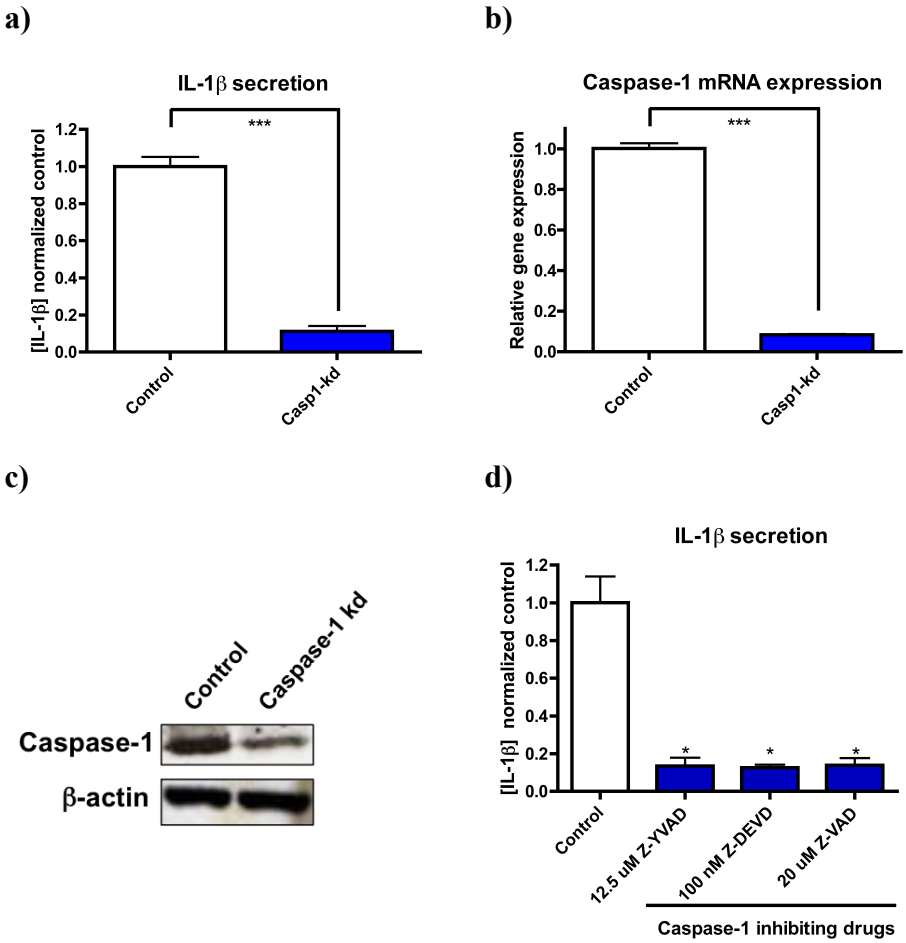


Figure 24 – Caspase-1 dependent IL-1β secretion. IL-1β secretion is dependent on its pro-form processing by Caspase-1. a) Caspase-1 knockdown by RNAi almost completely abolish IL-1β secretion after 24 hr *E.coli* challenge. b,c) Caspase-1 knockdown efficiency was assayed by qRT-PCR (b) - to measure its mRNA expression upon knockdown ; and by western-blot (c)- to measure knockdown efficiency at the protein level. (d)Targeting Caspase-1 with different inhibitors (Z-VAD, Z-DEVD and Z-YVAD) decreased IL-1β secreted levels. β-actin was used as a protein loading control (t-student: *p<0.05, ***p<0.0001).

Several methods have been described to measure Caspase-1 activity, although propably the most sensitive relies on using analogs of Caspase-1 substrates that irreversibly bind to the active Caspase-1 catalytical site. These

analogs can be fused to fluorophores (such as Carboxyfluorescein, FAM) and its fluoresence can be measured by spectophotometry or by FACS analysis. By using one of these comercial available probes for Caspase-1 activity detection (FLICA® from Immunochemistry®), we have measured Caspase-1 activity by FACS at different time points upon *E.coli* challenge. Figure 25a clearly shows that Caspase-1 activation increases over time after *E.coli* challenge. In order to unveil if Caspase-1 expression is also induced after an *E.coli* stimuli, we have measured Caspase-1 mRNA expression by qRT-PCR. Augmented Caspase-1 mRNA expression is detected after *E.coli* challenge, as shown at Figure 25-b.

a) **b)**

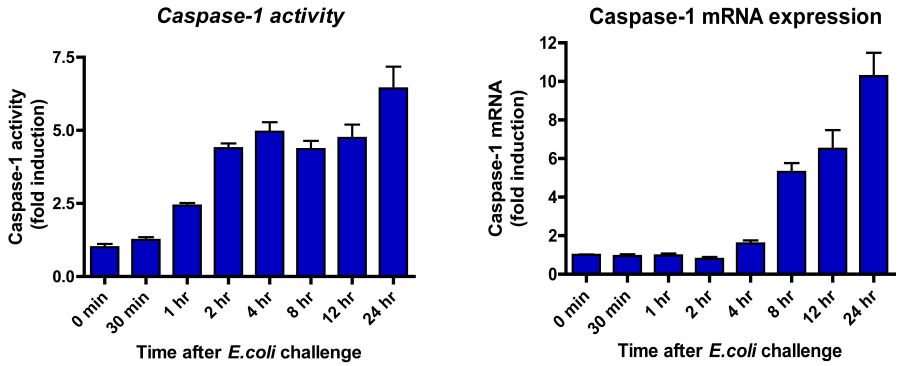


Figure 25 – Caspase-1 mRNA expression and activity. a) Caspase-1 activity measured by FACS using Caspase-1 substrate (FLICA). Caspase-1 activity is increased over time following an *E.coli* challenge. b) Caspase-1 mRNA expression is induced upon *E.coli* challenge, as measured by qRT-PCR.

These results confirm that Caspase-1 is required for IL-1β secretion upon *E.coli* challenge. As demonstrated using Caspase-1 inhibitors, its activity is mandatory in order to process IL-1β into its mature form. In addition, increased Caspase-1 mRNA expression and activity was indeed detected in THP-1 cells upon *E.coli* challenge.

3.1.3 – Identification of the Inflammasome involved in IL-1β secretion

We have previously shown that in order to be secreted, IL-1β needs to be processed by Caspase-1. However, as discussed in Chapter 1.1, Caspase-1 itself needs to be activated by the Inflammasome in order to become an active protease (*Martinon et al.*, 2002; reviewed in *Martinon et al.*, 2009). Several Inflammasome complexes have been identified, such as the NALP3, IPAF, AIM2 (Figure 7), as being activated by different PAMPs (reviewed in *Martinon et al.*, 2009). Thus, to better characterize our model of study, we decided to develop several experiments to identify the major Inflammasome involved in IL-1β secretion following an *E.coli* challenge.

However, before performing such experiments, we decided to look if other stimuli, in addition to *E.coli*, are required for maximum IL-1β secretion after an *E.coli* challenge. Several studies report the different Inflammasome activation requirements by using either monocytes or macrophages, such as the usage of extracellular ATP in the case of macrophages studies (*Walev et al.*, 1995; *Kahlenberg et al.*, 2004; *Pelegrin et al.*, 2006; *Duncan et al.*, 2007; *Netea et al.*, 2008). Furthermore, *Netea et al*. reported that THP-1 cells, in order to be able to secrete IL-1β, require an additional stimulus induced by ATP (reviewed in *Netea et al.*, 2008; *Netea et al.*, 2010). However, in our previous studies, as shown in Figure 22-b, we have observed IL-1β secretion by THP-1 cells after *E.coli* challenge, with no need to add extracellular ATP. Even though, we decided to test that hypothesis.

3.1.3.1 - Extracellular ATP addition is not required for IL-1β secretion

The early observations by *Hogquist, Perregaux and Griffiths* (*Hogquist et al.*, 1991; *Perregaux et al.*, 1994; *Griffiths et al., 1995*) clearly show that ATP is a strong IL-1β releasing agent. Moreover, a massive efflux of potassium (K^+) is triggered by ATP, strongly suggesting that ATP activates the Inflammasome by decreasing intracellular K^+ levels (*Perregaux et al.*, 1994; *Pétrilli et al.*, 2007). Exogenous added ATP-driven maturation and release of IL-1β was then identified to be specifically mediated by an P2X7 receptor dependant manner (*Ferrari et al.*, 1997; *Solle et al.*, 2001).

In order to assess the role of ATP in our model we have decided to perform an experiment where in addition to 24 hr *E.coli* challenge, THP-1 cells were also stimulated with 3 mM of extracelullar ATP. As can be observed in Figure 26, using ATP, in addition to *E.coli.* showed an increase in the amount of IL-1β secreted, although not statistically significant.

In addition to the role of ATP, the role of the P2X7 receptor in Inflammasome activation induced by *E.coli* challenge was also assessed. Using a known P2X7 receptor inhibitor, KN-62 (*Baraldi et al.,2003; Friedle et al., 2010*), a remarkable decrease in IL-1β secretion was observed in treated cells, when compared to IL-1β levels secreted by non-treated cells (Figure 26). Therefore we could conclude that P2X7 receptor plays a role in IL-1β secretion after *E.coli* challenge, however no extracellular ATP addition is needed in our model.

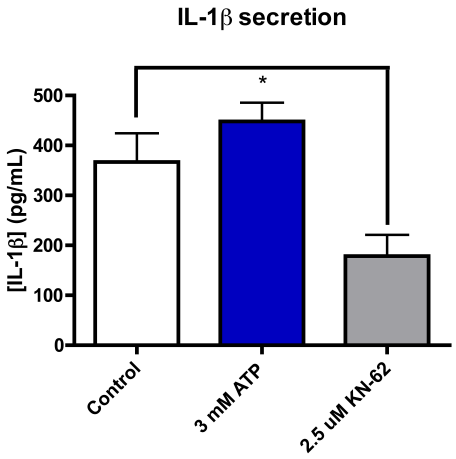


Figure 26 - Role of ATP and P2X7 in IL-1β secretion after 24 hr *E.coli* challenge. THP-1 cells were challenged with *E.coli* for 24 hr either in the presence or absence of a P2X7 receptor inhibitor (KN-62) or extracellular ATP. IL-1β secretion was assayed by ELISA. Cells that were challenged with *E.coli* and then exogenously treated with 3 mM of ATP (for 30 min before the end of the assay) showed an increase in IL-1β secretion, although not statistically significant. In opposition, THP-1 cells treated with KN-62 showed a statistically significant decrease in IL-1β secretion after 24 hr of *E.coli* challenge. (t-student: *$p<0.05$)

3.1.3.2 - NALP3 Inflammasome regulates IL-1β secretion

In order to identify the major Inflammasome involved in Caspase-1 activation and subsequent IL-1β secretion upon *E.coli* challenge, we decided to perform an RNAi targeted screen. We used a subset of the TRC library enriched in shRNAs that target all the NLRs described so far and several CARD-containing proteins (Table S1). Briefly, as described in Chapter 2 (Figure 19), we infected THP-1 cells with a lentiviral vector encoding the shRNA of interest. After selection of the infected cells, we split the total cells into two, in order to evaluate

the number of cells by Alamar Blue® assay and to challenge the remaining cells with the 4% PFA-fixed *E.coli*. After challenge, cell supernatants were assayed for the presence of secreted IL-1β by ELISA. All concentrations were then normalized by the number of cells and then compared with the IL-1β secreted levels obtained with control (Scramble) shRNA (as represented in the equation below).

$$z\,score = \log\left[\frac{\left(\frac{[IL-1\beta]}{cell}\right)shRNA\,Gene}{\left(\frac{[IL-1\beta]}{cell}\right)shRNA\,Control}\right]$$

The z-score values can then be represented in a more visual "friendly" way if organized from the lowest to the highest z-score values (Figure 27). As can be seen in Figure 27, the values follow a continuous curve, where the lowest values are depicted for positive regulator candidates and the highest values for negative regulator candidates. A candidate was considered a negative regulator (represented in blue box) if at least 2 out of 5 shRNA targeting the same gene showed a score higher than 1.8 standard deviations above the average of the control. In opposition, a positive regulator candidate (represented in red box) was depicted if at least 2 out of 5 shRNA targeting the same gene showed IL-1β secreted levels inferior to 1.8 Standard deviations to the average of the control. All the candidates are listed on Table 4. Since we were interested in the identification of the major Inflammasome activated after *E.coli* challenge, we have looked more carefully to the positive regulator candidates, as the ones where knocking down showed decreased IL-1β secretion.

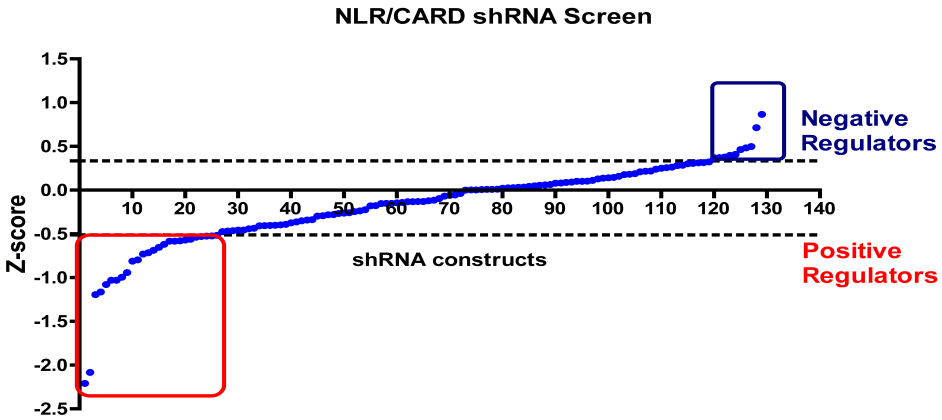


Figure 27 - Identification of NLRs and CARD domain containing proteins with a role in IL-1β secretion. 29 NLR/CARDs genes were targeted in THP-1 cells by lentiviral shRNA delivery, with an average of five-fold coverage per gene. After 24 hr *E.coli* challenge, IL-1β secretion was measured by ELISA. After normalization of IL-1β secreted levels, several genes showed a clear phenotype either by increasing or decreasing IL-1β secretion, therefore named negative (in blue) and positive regulators (in red), respectively.

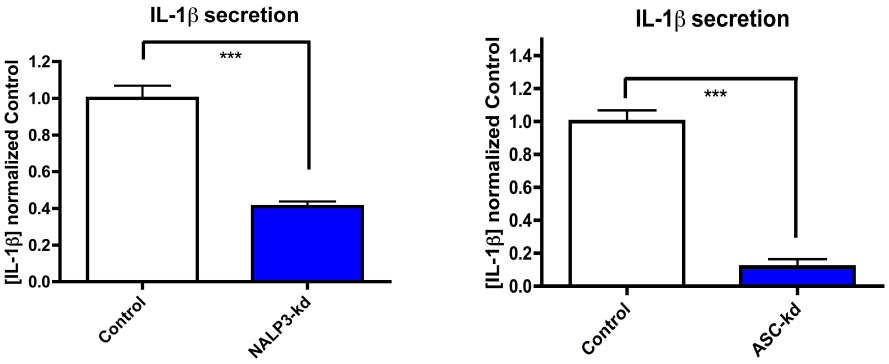
Table 4 – List of NLR/CARD candidates to play a role in IL-1β secretion after *E.coli* challenge obtained from the RNAi based screen.

Positive Regulators	Negative Regulators
ASC	NALP4
CARMA1	NALP9
NALP12	
NALP3	
NALP8	
VISA	

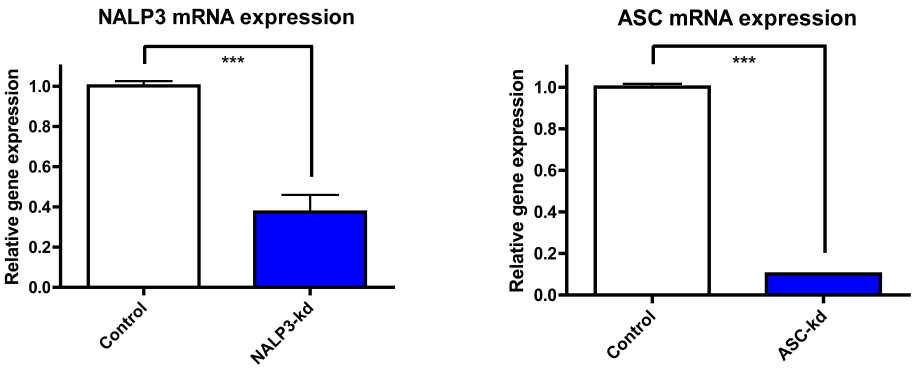
Among the positive regulator candidates, NALP3 and ASC, two key components of NALP3 Inflammasome (reviewed in *Martinon et al.*, 2009), showed the strongest phenotype. The levels of IL-1β secreted after knocking down NALP3 and ASC and posterior 24 hr *E.coli* challenge are represented at Figure 28-a. In order to validate the results, the levels of knockdown of NALP3 and ASC were evaluated. As depicted in Figure 28-b, a remarkable decreased mRNA expression of both, NALP3 and ASC, was measured upon targeting these two

genes by RNAi. In addition, the protein levels of these two major components of the NALP3 Inflammasome were also assayed. Decreased expression of both proteins is also observed after knockdown by RNAi (Figure 28-c).

a)



b)



c)

Figure 28 – NALP3 Inflammasome regulates IL-1β secretion. THP-1 cells were infected with lentiviral vectors encoding shRNA specific for NALP3 or ASC. a) IL-1β secretion after 24 hr *E.coli* challenge. Decreased IL-1β secretion after knockdown of NALP3 and ASC. The efficiency of knockdown of the candidates was assayed by qRT-PCR (b) and by Western-blot (c). β-actin was used as a protein loading control (t-student: ***$p<0.0001$).

In sum, our results show that the NALP3 Inflammasome plays a major role in IL-1β secretion by THP-1 cells following an *E.coli* challenge. However, we cannot exclude that other Inflammasomes may be involved in the regulation of this Inflammatory response. Further experiments would be required to prove this assumption.

3.2 - Alternative Splicing and the regulation of IL-1β secretion

Alternative Splicing has gained an extraordinary attention with the completion of the Human Genome Project, where fewer genes were found to exist than it was initially predicted (*Lander et al.*, 2001; *Venter*, 2001). This process has been extensively studied in the past years due to its role in the generation of proteome diversity and in the regulation of several cellular processes (reviewed in *Black*, 2000; *Smith et al.*, 2000; *Graveley*, 2001). As described extensively on Chapter 1.2, the number of genes known to undergo AS has been increasing over time, and at the moment it is estimated that around 92-94% of the genes are alternatively spliced (*Pan et al.*, 2008; *Wang et al.*, 2008). Simultaneously, the number of cellular processes regulated by AS has also increased. Focusing on the IL-1β pathway, different isoforms of several genes are already reported to play important roles in the regulation of this Inflammatory response (Figure 10, reviewed in Chapter 1.1.3). However, besides the identification of numerous isoforms of "key-components" of this response, little is still known about the total number of genes regulated by AS and their functions in the regulation of IL-1β secretion.

As extensively mentioned in Chapter 1.2.1.1, AS is tightly regulated, where different cellular pathways and molecules have been unveiled as important regulators of this process. Subtle variations in the regulation of Splicing have been described to be involved in different, and sometimes opposite, outcomes of certain cellular responses, and in certain cases leading to disease (reviewed in *Black*, 2003; *Tazi et al.*, 2008; *Cooper et al.*, 2009). As an example, the work by *Paronetto et al.* elegantly shows that modulating the concentration of several SFs, different

isoforms of the Bcl-x gene are generated (*Paronetto et al.*, 2007). Furthermore, the different isoforms have completely opposite effects in the regulation of apoptosis, from an anti-apoptotic to a pro-apoptotic effect if the long or the short isoforms are generated, respectively (reviewed in *Boise et al.*, 1993; *Youle et al.*, 2008). Thus, we have decided to follow a similar concept, changing the expression of different SFs and evaluating the impact in IL-1β secretion.

3.2.1 - Identification of Splicing Factors with a role in IL-1β secretion

In an attempt to identify the SFs that could play a role in the secretion of IL-1β after *E.coli* challenge, we have performed an RNAi-based screen. We screened 425 genes (listed at Table S2) that were previously reported to be important players either in Spliceosome assembly, Splicing or proteins found to interact with Spliceosome components (*Longman et al., 2000*). The screening outline and the analysis performed was similar to what was already described in Chapter 3.1.3, although, in spite of normalizing the values with the control shRNA, values were normalized against the mean of IL-1β per cell in all the constructs for all the genes assayed in each plate tested. A similar type of analysis was already performed in other published studies (*Oberdoerffer et al., 2008*).

The screening results are shown in Figure 29. Genes were considered candidates if at least 2 out of 5 shRNA targeting the same gene showed IL-1β secretion superior or inferior to 1.5 Standard Deviations to the average of the control. After a secondary screen, and several rounds of phenotypic validation, 30

genes were considered candidates, 20 positive regulators and 10 negative regulators of IL-1β secretion (Table 5).

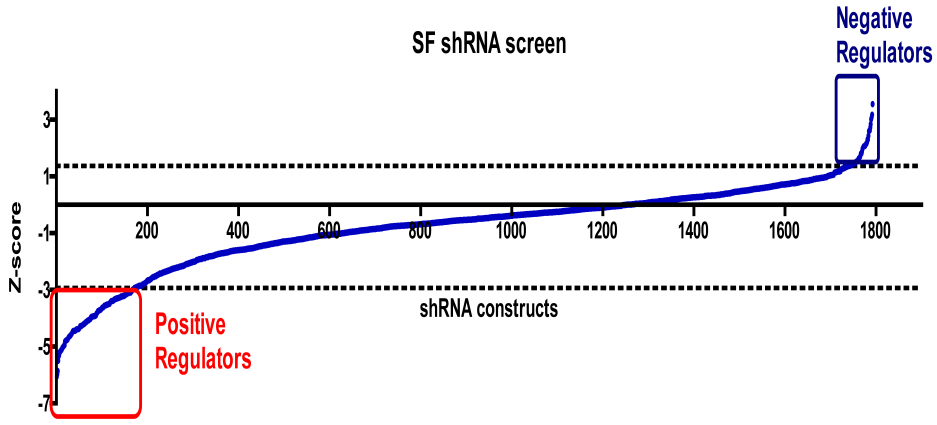


Figure 29 - Identification of Splicing Factors with a role in IL-1β secretion by RNAi based screen. 425 Splicing Factors (SFs) were targeted in THP-1 cells by lentiviral shRNA delivery, with an average of five-fold coverage per gene. After 24 hr *E.coli* challenge, IL-1β secretion was measured by ELISA. Several of the genes tested showed a clear phenotype either by increasing or decreasing IL-1β secretion upon knockdown, therefore named negative (in blue) and positive regulators (in red), respectively.

As a representative example of two SF candidates that play a role in IL-1β secretion, Figure 30 shows the phenotypes observed for a positive and a negative regulator candidates, HMGB1 and SFRS3, respectively. As can be seen, there are at least 2 out of 5 shRNA constructs with a similar phenotype upon knockdown, either increasing or decreasing IL-1β secretion, therefore negative (in blue) and positive (in grey) regulators candidates, respectively. All the other candidates are listed on Table 5.

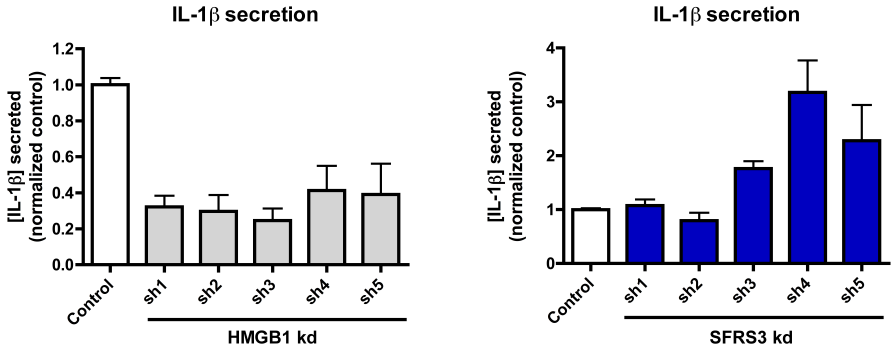


Figure 30 – Splicing Factor candidates to play a role in IL-1β secretion. Five different shRNA were used to target each SF candidate in THP-1 cells. If at least 2 out of 5 showed an increase or decrease in IL-1β secretion over the 1.5 StDev, it was considered a candidate. Therefore, as an example, HMGB1 was depicted as a positive regulator candidate (in grey) and SFRS3 as a negative regulator candidate (in blue) due to their decreased/increased effect in IL-1β secretion upon knockdown and posterior 24 hr *E.coli* challenge, respectively.

Table 5 – List of Splicing Factor candidates to play a role in IL-1β secretion obtained from the RNAi based screen.

Positive Regulators	Negative Regulators
CLK1	AKAP8
CLK4	GNB2L1
CUGBP2	HYPC
CWF19L1	MFAP1
DDX54	PPIE
DHX38	PPIH
EIF3S10	SFPQ
GTF2LI	SFRS1
HMGB1	SFRS3
HNRPM	TET1
KHSRP	
KIN	
LSM7	
PABPN1	
SF3A3	
SFRS2	
SFRS12	
SKIV2L2	
SNIP1	
ZC3H13	

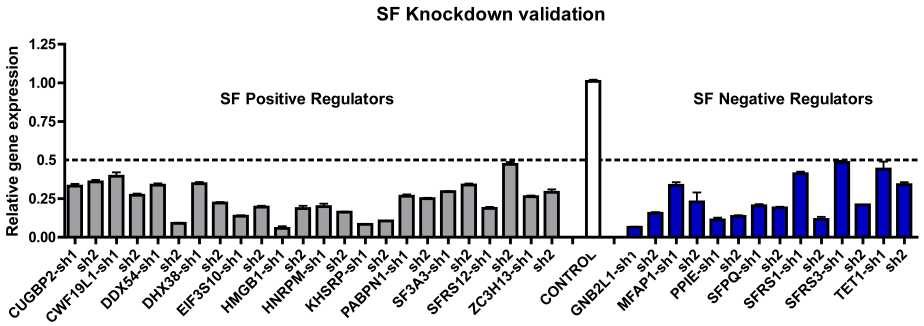
3.2.1.1 - Validation of candidates obtained in the RNAi-based screen

After performing the RNAi-based screen, in order verify if the phenotypes observed were really due to the knockdown of a certain gene, mRNA expression of all the target genes was evaluated by qRT-PCR analysis. The knockdown efficiency was then calculated by comparing the expression of the gene of interest in cells infected with the control shRNA and the shRNA targeting the gene of interest. A candidate was considered validated if at least 2 out of 5 shRNA targeting each gene, that showed a phenotype in IL-1β secretion, have a knockdown efficiency over 50% at mRNA level . The total list of validated genes is present at Table 6 and their respective mRNA expression and IL-1β secretion profiles are present in Figures 31-a and b, respectively. Therefore, according to the criteria defined, the rate of validation of the SF candidates was 63%.

Table 6– List of the validated Splicing Factor candidates

Positive Regulators	Negative Regulators
CUGBP2	GNB2L1
CWF19L1	MFAP1
DDX54	PPIE
DHX38	SFPQ
EIF3S10	SFRS1
HMGB1	SFRS3
HNRPM	TET1
KHSRP	
PABPN1	
SF3A3	
SFRS12	
ZC3H13	

a)



b)

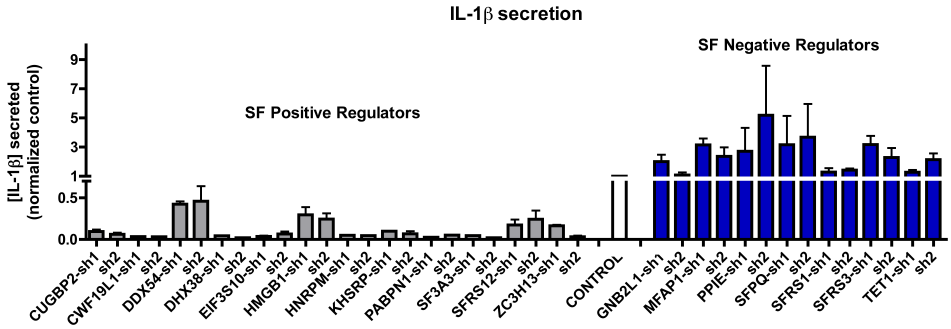


Figure 31 - Knockdown validation of SF candidates obtained from the shRNA screen. THP-1 cells were infected with a lentiviral vectors encoding the shRNA molecules specific for each SF candidate. a) The knockdown efficiency of the SF candidates was assayed by qRT-PCR. The expression of a certain gene was normalized with the expression of the same gene in the control. b) IL-1β secretion phenotype, after 24 hr *E.coli* challenge, of the different shRNAs used to target each SF candidate. IL-1β secreted levels were normalized with the levels observed in the control shRNA.

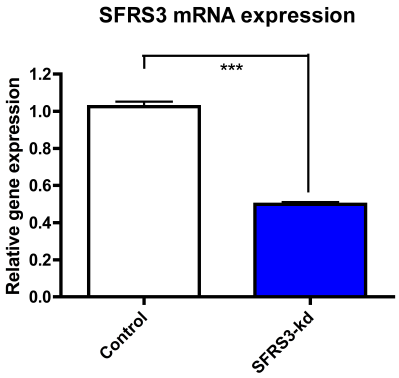
From all the 19 candidates validated, we selected 2 of the Negative Regulators (SFRS1 and SFRS3) and the Positive regulator, High-mobility group box-1 (HMGB1), for further studies.

SFRS1 and SFRS3 are two "bona-fide" members of the SR protein family of SFs (reviewed in Chapter 1.2.1.1 and reviewed in *Long et al.*, 2009). SFRS1 (ASF/SF2) was the first SF to be identified (*Krainer et al.*, 1990a) and is probably one of the most studied SFs (reviewed in *Long et al.*, 2009). SFRS1 has been shown to play a role in several cellular processes, such as constitutive and alternative splicing (*Ge et al.*, 1990; *Krainer et al.*, 1990b) transcription (*Li et al.*, 2005a; *Li et al.*, 2007), translation (*Michlewski et al.*, 2008; reviewed in *Li et al.*, 2005a*;Long et al.*, 2009). Moreover, the role of SFRS1 in several diseases has also been studied in great detail, such as cancer (*Fischer et al.*, 2004; *Karni et al.*, 2007; *Stickeler et al.*, 1999), AIDS (*Asang et al.*, 2008), and more recently it was shown that SFRS1 may play a role in Inflammation (*Xiong, 2006*). The work from *Xiong et al.* shows that patients with an Inflammatory condition, inflamed muscle, have a remarkable decreased ASF/SF2 expression (Note: ASF/SF2 is the protein encoded by the SFRS1 gene). In addition, *in vitro* stimulation of Mouse C2C12 skeletal muscle myoblasts with a pro-inflammatory mediator, TNF-α, showed a similar phenotype, thus unveiling a possible role of ASF/SF2 in the regulation of an Inflammatory response. The other SR protein, SRp20 (Note: SRp20 is the protein encoded by the SFRS3 gene) is far less studied than ASF/SF2. However besides its role in Splicing, SRp20 have also been demonstrated to be important in other cellular processes, such as nucleocytoplasmic export of mRNA (*Huang et al.*, 2001; *Huang et al.*, 2003; *Huang et al.*, 2004) or in translation (*Bedard et al.*, 2007). The IL-1β secretion phenotype observed after knockdown this SF was one of the most remarkable and consistent found during the RNAi based screen (Figure 31-b). Therefore we decided to further study its role in the regulation of IL-1β secretion, in addition to SFRS1.

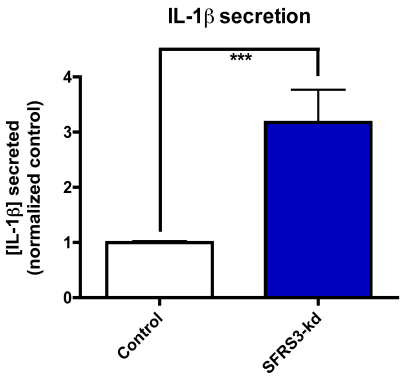
In Figure 32 are represented, for each gene, the different values (knockdown and IL-1β secretion) for the shRNA construct used on further studies. In addition to the mRNA knockdown validation, the protein levels of these 2 SF was also

assayed by Western blot. As can be seen in Figure 32-e, in agreement to what was observed at the mRNA level, the protein levels of both SF are decreased upon knockdown. In addition, knocking-down one SF did not show any impact in the expression of the other candidate (Figure 32-f).

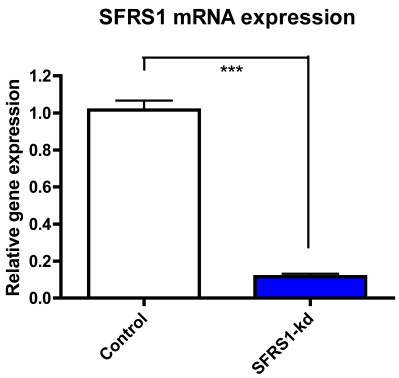
a)



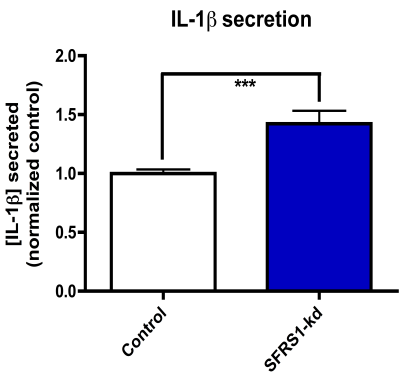
b)



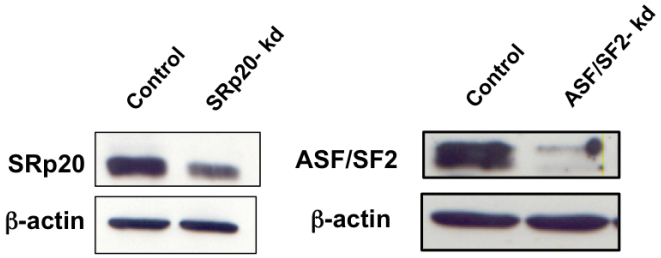
c)



d)



e)



f)

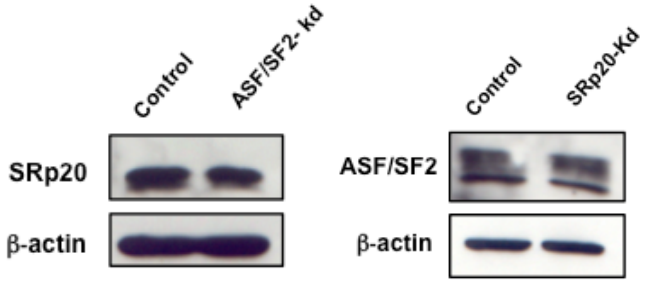


Figure 32 – Knockdown validation of SFRS1 and SFRS3. THP-1 cells were infected with Lentiviral vectors encoding shRNAs to target SFRS1 and SFRS3. The efficiency of knockdown of the SF candidates was assayed at mRNA level by qRT-PCR (a and c) and at protein level (e), by Western-blot. b, d) IL-1β secretion phenotype for the shRNA used to target each SF candidate after 24 hr *E.coli* challenge. f) SRp20 protein expression upon ASF/SF2 knockdown and vice-versa. SRp20 and ASF/SF2 are the proteins encoded by the genes SFRS3 and SFRS1, respectively. β-actin was used as a protein loading control (t-student: ***$p<0.0001$).

In addition to the Negative Regulator SF candidates, we decided to further validate the Positive Regulator SF candidate HMGB1. The role of HMGB1 in Splicing or Splicing Regulation have not yet been unveiled, however it was found as a putative Splicing Regulator protein during a Proteomic Analysis of the Spliceosome (*Barbosa-Morais*, 2005). In opposition, an important role in Inflammation has been attributed to this candidate. The HMGB1 has been identified to be actively secreted by stimulated macrophages and monocytes and to act as potent pro-inflammatory mediator (*Wang et al.*, 1999; reviewed in *Erlandsson Harris et al.*, 2004). Furthermore, a report by *Mouri et al.* shows that HMGB1 can regulate IL-1β production by the transactivation of its promoter (*Mouri et al.*, 2007). In a similar manner to what was performed for SFRS1 and SFRS3, HMGB1 mRNA and protein expression was evaluated upon knockdown. Targeting THP-1 cells with the lentiviral vectors encoding an HMGB1 specific shRNA reduced its expression at mRNA (Figure 33-a) and protein levels (Figure 33-c), and showed a clear reduction in IL-1β secretion upon 24 hr *E.coli* challenge (Figure 33-b).

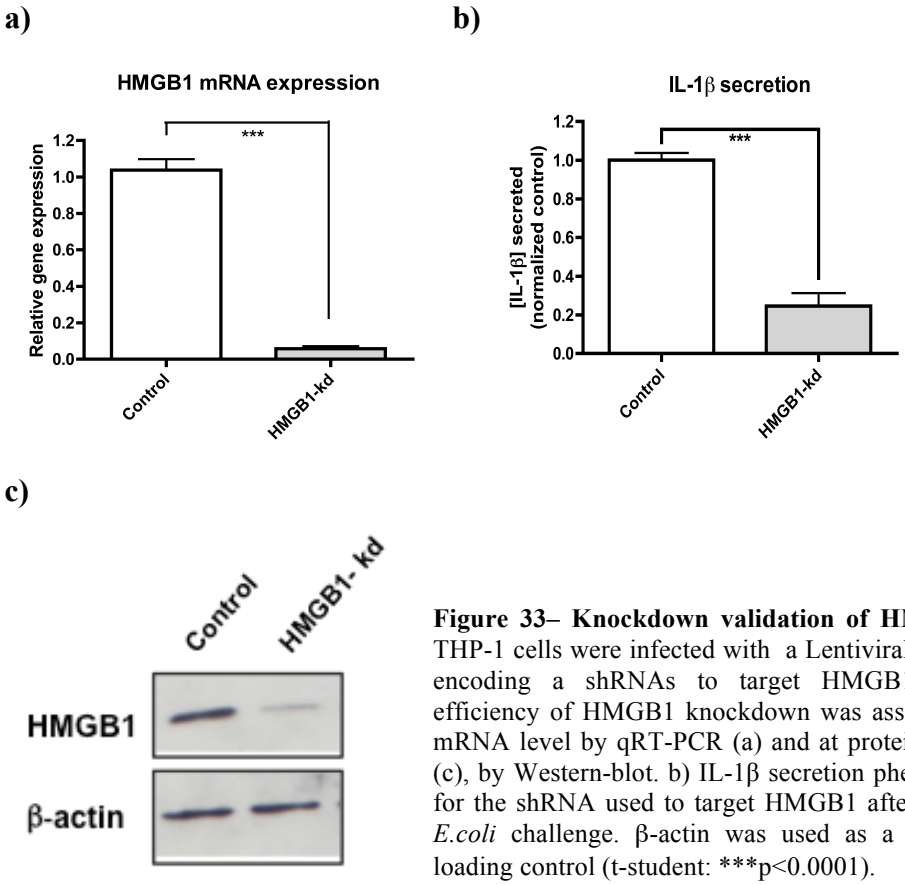


Figure 33– Knockdown validation of HMGB1. THP-1 cells were infected with a Lentiviral vector encoding a shRNAs to target HMGB1. The efficiency of HMGB1 knockdown was assayed at mRNA level by qRT-PCR (a) and at protein level (c), by Western-blot. b) IL-1β secretion phenotype for the shRNA used to target HMGB1 after 24 hr *E.coli* challenge. β-actin was used as a protein loading control (t-student: ***p<0.0001).

3.2.2 - Validation of SFRS1 and SFRS3 as Negative Regulators of IL-1β secretion

In an attempt to validate the Negative roles of SFRS1 and SFRS3 in the regulation of IL-1β secretion after a bacterial challenge, we performed other

complimentary studies, either using drugs to target the two SFs or by overexpression experiments.

3.2.2.1 – Drugs targeting SFRS1 and SFRS3 show a similar Negative Regulator phenotype

The strong connection between Splicing and disease has "boosted" the interest of finding drugs that may be used to modulate certain cellular responses by targeting some key-components in Splicing, such as SFs. One such example is the work of *Keriel* and colleagues (*Keriel et al., 2009*) where several drugs were screened for their suppressive effect in the production of ASF/SF2 Splicing dependent retroviral proteins, therefore promoting protection against retrovirus pathogenesis. Other study, by *Stoilov et al.* (*Stoilov et al.*, 2008), have identified Digoxin as a compound capable of modulating microtubule-associated protein tau (MAPT) exon 10 Splicing by decreasing the expression of SRp20. Therefore, to confirm the phenotypes observed by RNAi, we have used drugs that inhibit ASF/SF2 dependent Splicing, kindly provided by Dr. *Jamal Tazi*, and Digoxin to target SRp20. Briefly, THP-1 cells were treated with drugs or DMSO (as control) and further challenged with *E.coli* for 24 hr. Supernatants were collected and IL-1β concentration assayed by ELISA. Inhibiting ASF/SF2 dependent Splicing using BO-3 and BO-26 compounds, increased the levels of IL-1β secreted after 24 hr *E.coli* challenge, when compared with control (Figure 34-a). A similar result was found when SRp20 expression was decreased using Digoxin (Figure 34-b). Indeed, to confirm the downregulation of SRp20 expression upon digoxin treatment, an immunoblot was performed in Digoxin treated cell lysates. As can be seen in Figure 34-d, SRp20 expression is decreased upon treatment; moreover, the positive

effect of digoxin treatment on IL-1β secretion is inversely correlated with SRp20 expression (Figures 32 c and d).

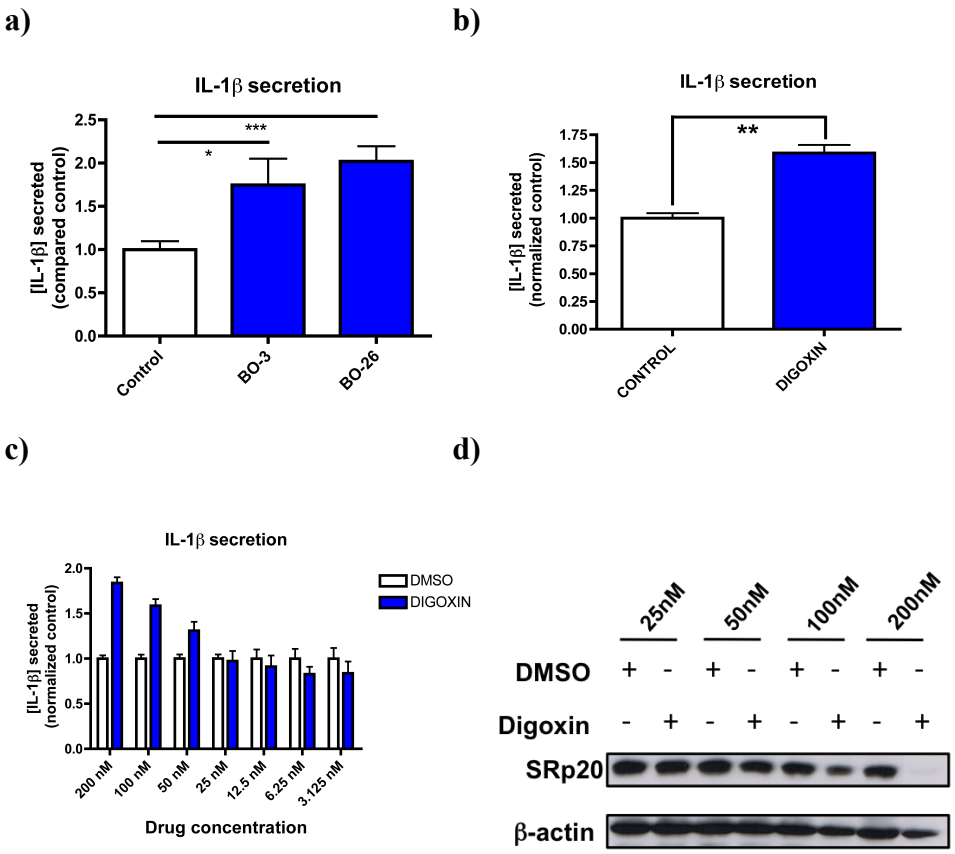


Figure 34 – The effect of drugs targeting SFRS1 and SFRS3 on IL-1β secretion. a) BO-3 and BO-26, two compounds that abolish SFRS1 dependent Splicing events were used to treat THP-1 cells previous to *E.coli* challenge. Increased IL-1β secretion was observed upon targeting SFRS1 using both drugs. b) In a similar way, Digoxin treatment increased IL-1β secretion after 24 hr *E.coli* challenge. c) Moreover, the increased IL-1β secretion is dependant on the digoxin dose used. d) Digoxin treatment reduces the expression of SRp20 in a dose dependant manner. SRp20-protein encoded by SFRS3. β-actin was used as a protein loading control (t-student: *p<0.05,**p<0.005,***p<0.0001).

3.2.2.2 – Overexpression of SFRS1 and SFRS3 decreases IL-1β secretion

The results obtained using RNAi or drugs targeting SFRS1 and SFRS3, clearly show that either decreasing both SFs expression or impairing their Splicing dependent reactions, increased IL-1β secretion. Thus, in an attempt to counteract this phenomena and further prove the negative regulator effect of these two SFs, we decided to overexpress both candidates and evaluate the impact of the overexpression in IL-1β secretion. We have generated lentiviral vectors (Figures 20, 21, S1 and S2) to promote the overexpression of these SFs and then analyze their role in IL-1β secretion induced by *E.coli* challenge. Briefly, as mentioned in Chapter 2.2.9, SFRS1 and SFRS3 were cloned into a lentiviral vector (pLenti6/V5-DEST) and viruses were produced in 293T cells. The lentiviruses generated were then used to infect THP-1 cells. After selection with Blasticidin®, the infected cells were challenged with *E.coli* for 24 hr and supernatants were collected and assayed for IL-1β levels by ELISA. As can be seen in Figure 35, overexpressing SFRS1 or SFRS3 resulted in a remarkable decrease in IL-1β secretion, as compared with the control. The overexpression was confirmed by immunoblot to detect the V5 epitope that is fused to the protein of interest (Figures 21 and S2) as shown in Figure 35-b.

Thus, these results further substantiate the role of SFRS1 and SFRS3 as negative regulators of IL-1β secretion.

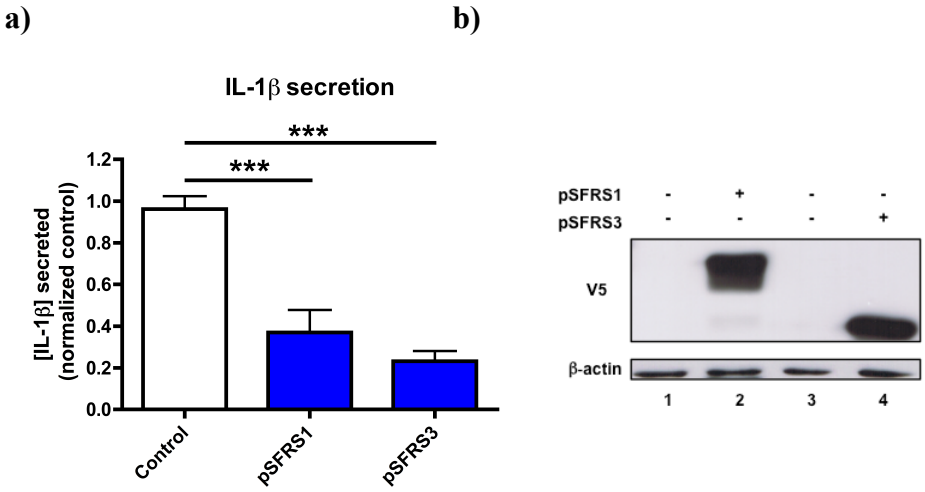


Figure 35 – Overexpression of SFRS1 and SFRS3. Lentiviruses were used to overexpress SFRS1 and SFRS3 in THP-1 cells. After infection cells were challenged with *E.coli* and IL-1β concentration was measured in the supernatants by ELISA. a) Overexpression of SFRS1 and SFRS3 decreased IL-1β secretion after 24 hr *E.coli* challenge, as compared to control. b) Western blot detection V5-epitope tagged SFRS1 and SFRS3 proteins. pSFRS1 and pSFRS3 are the lentivirus encoding the SFRS1 and SFRS3 V5 tagged proteins. Lanes 1 and 3-control non-infected cells. Lanes 2 and 4 cells overexpressing ASF/SF2 or SRp20, respectively. β-actin was used as protein loading control (t-student: ***p<0.0001).

3.2.3 - SFRS1 and SFRS3 effects on IL-1β secretion pathway

Interleukin-*1β* secretion is the outcome of a two-step mechanism, Production and Processing, as described in Chapter 1.1.2 (Figure 9, reviewed in *Creagh et al.*, 2006; *Dinarello*, 2009; *Martinon et al.*, 2009). To dissect the contributions of SFRS1 and SFRS3 in the regulation of both steps required for IL-1β secretion we have developed several assays. We have measured IL-1β mRNA expression after SFRS1 and SFRS3 knockdown, as a quantitative method to

observe the impact in the Production step. To elucidate their role in the Processing step, we took advantage of an indirect method to detect Inflammasome activation, by measuring Caspase-1 activity.

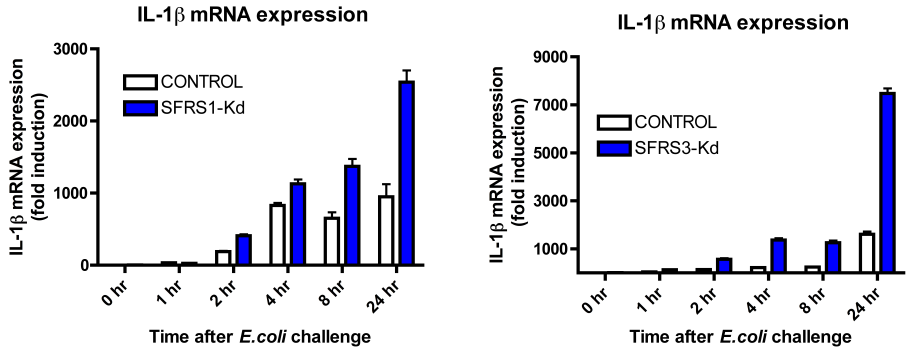
3.2.3.1 - SFRS1 and SFRS3 regulate IL-1β mRNA expression

One of the key elements in the production of IL-1β is the activation of the NF-kB transcription factor (*Dinarello*, 2009). Upon activation, NF-kB is translocated into the nucleus and promotes the transcription of several pro-inflammatory cytokines, such as IL-1β and IL-8 (*Dinarello*, 2005; *Remick*, 2005). In addition to IL-1β, IL-8 expression and secretion in THP-1 cells was already mentioned to be induced upon *E.coli* challenge (Figures 22 and 23). Therefore, we decided to measure both cytokines mRNA expression as an indirect, however quantitative method, of NF-kB activation and IL-1β production.

Interleukin-1β and IL-8 mRNA expression was measured by qRT-PCR at different time points after THP-1 challenge with *E.coli*. Once more we could observe that the mRNA expression of both cytokines is induced by *E.coli* challenge (Figure 36). However, upon SFRS1 and SFRS3 knockdown, their mRNA expression is much higher when compared with control (Figure 36). Moreover, the increased mRNA expression of both IL-1β and IL-8 is much higher in cells targeted for SFRS3 than in SFRS1 knocked-down cells (Figure 36). This observation correlates with the higher secretion of IL-1β in SFRS3 knocked-down cells as compared with cells with decreased SFRS1 expression (Figures 32-b and d; S4-a and S4-b).

Interleukin-1β concentration was also measured in the cell supernatants at the different time points following *E.coli* challenge. Increased IL-1β secretion is already detected 4hr post-challenge upon SFRS1/SFRS3 knockdown as compared with control cells (Figures S4-a and b).

a)



b)

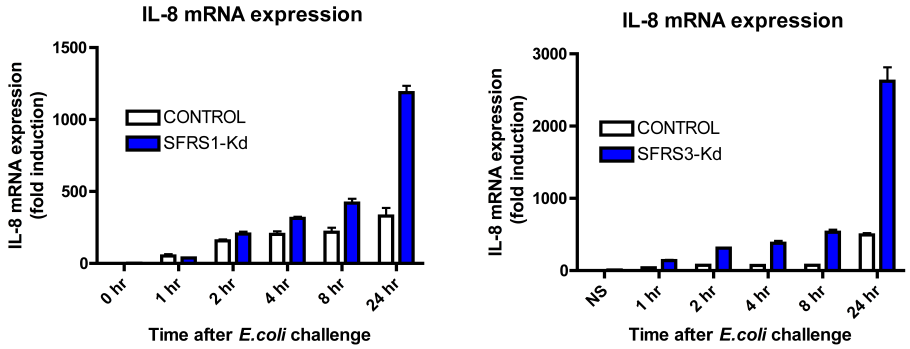


Figure 36 – Kinetics of IL-1β and IL-8 mRNA expression upon SFRS1/SFRS3 knockdown. THP-1 cells were infected with lentiviral vectors encoding specific SFRS1 or SFRS3 shRNA. After infection cells were challenged with *E.coli* for different times. a) Increased IL-1β mRNA expression, detected by qRT-PCR, in cells where SFRS1 and SFRS3 were down regulated. Higher induction was found in SFRS3 knocked-down cells, as compared with control and SFRS1 RNAi targeted cells. b) Similar to the results obtained for IL-1β, IL-8 mRNA expression was also increased in SFRS1 and SFRS3 knocked-down cells, with higher values in the last one.

The Positive Regulator candidate HMGB1 has been recently reported to be an endogenous Danger Signal (DAMP), therefore capable of inducing Inflammation (reviewed in *Erlandsson Harris et al.*, 2004; *Klune et al.*, 2008). Moreover, it was shown that HMGB1 can act as a transactivator of IL-1β promoter, therefore being involved in IL-1β production (*Mouri et al., 2007*). As a proof of principle, we performed a similar approach to what was done previously, for SFRS1 and SFRS3, to measure IL-1β mRNA expression upon HMGB1 knockdown. The results confirmed the positive role of HMGB1 in IL-1β production, as can be seen in Figures 37 and S4-c, where decreased IL-1β mRNA expression and secretion occurs in HMGB1 RNAi targeted cells.

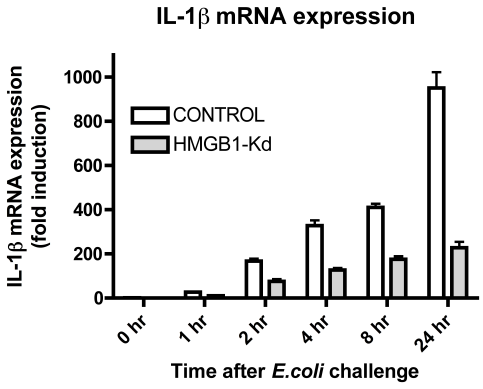


Figure 37 - Kinetics of IL-1β mRNA expression in HMGB1 knockdown cells. THP-1 cells were infected with lentiviruses encoding a specific HMGB1 shRNA. Infected cells were further challenged with *E.coli* for different time points. qRT-PCR analysis shows that IL-1β mRNA expression is decreased in cells where HMGB1 was knocked-down, as compared with control.

All these results prompted us to, besides validating our approach to find SFs with a role in IL-1β secretion, to conclude that SFRS1 and SFRS3 are negative regulators of IL-1β expression.

3.2.3.2 - SFRS3 regulates IL-1β processing

The second step necessary for IL-1β secretion is the Processing by the Inflammasome, in a Caspase-1 dependent manner, as shown in Figure 24 and extensively reviewed in Chapter 1.1.

Using Caspase-1 activity as an indirect method of Inflammasome activation detection, we have studied the role of both SFs in the second step of IL-1β secretion pathway. Briefly, as described in Chapter 3.1.2, Caspase-1 activity can be measured by FACS using FLICA, a fluorophore labeled Caspase-1 substrate that irreversibly binds to the catalytical center of active Caspase-1. We assayed Caspase-1 activity in cells knocked-down for SFRS1 or SFRS3 and further challenged with *E.coli* for 24 hr. The results obtained by FACS analysis were then normalized with the Caspase-1 activity measured in control infected cells stimulated with *E.coli*. As presented in Figure 38, SFRS1 does not play a role in this second step necessary for IL-1β secretion, as the levels of Caspase-1 activity are almost equal to control. In opposition, SFRS3 was found to be a negative regulator of Caspase-1 activation since increased levels of activation were detected upon knocking down this SF.

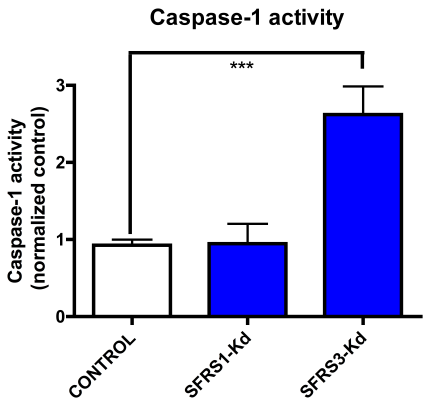


Figure 38 – Caspase-1 activity in SFRS1/SFRS3 RNAi targeted cells. THP-1 cells were infected with lentiviral vectors encoding specific SFRS1/3 shRNAs and further challenged with *E.coli* for 24 hr Caspase activation was measured by FACS. Cells knocked-down for SFRS3 showed an increased activation after 24 hr *E.coli* challenge, as compared with control and SFRS1 RNAi targeted cells. (t-student: ***p<0.0001).

These data showed that SFRS1 does not have an impact in this second step necessary for IL-1β maturation and release. In opposition, the increased Caspase-1 activity observed after knocking-down SFRS3, strongly suggests that this SF is involved in the regulation of the Inflammasome, however by an unknown mechanism.

3.2.3.2.1- SFRS3 regulates Caspase-1 mRNA expression

In order to study the underlying mechanism of SFRS3 regulation of Caspase-1 activity, we have measured the expression of the NALP3 Inflammasome components, to check if the augmented Caspase-1 activation could be related with increased expression of these molecules. In addition, we developed a preliminary approach to evaluate if other Inflammasome complexes are being activated upon SFRS3 knockdown.

The increased Caspase-1 activation observed upon knockdown of SFRS3, as compared with the control or SFRS1 knockdown suggested that Caspase-1 expression might be increased in cells targeted for SFRS3. qRT-PCR assays were performed to evaluate Caspase-1 mRNA expression kinetics in cells knocked-down for SFRS1, SFRS3 and in control infected cells. As can be seen in Figure 39 there is an increase in Caspase-1 mRNA expression in SFRS3 knocked-down cells, as compared to the expression levels in either SFRS1 and control cells. The increased levels are more striking 24 hr post-*E.coli* challenge, approximately 5-fold higher than in control-infected cells. This increased Caspase-1 mRNA expression in SFRS3, and not in SFRS1, RNAi targeted cells, correlates with the increased Caspase-1 activation observed, thus, suggesting that the increased Caspase-1 activity could indeed be due to increased Caspase-1 availability.

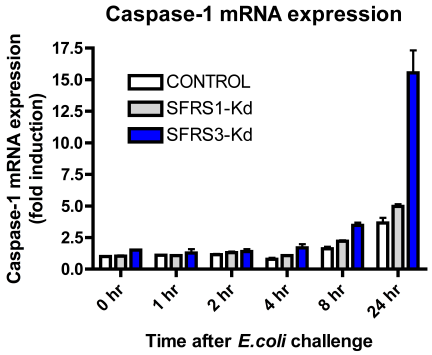


Figure 39 – Kinetics of Caspase-1 mRNA expression upon SFRS1 and SFRS3 knockdown. THP-1 cells were infected with lentiviral vectors encoding specific shRNAs targeting SFRS1 or SFRS3. Cells were further challenged with *E.coli* for different time points. Increased levels of Caspase-1 mRNA are found in cells knocked-down for SFRS3, as compared with SFRS1 RNAi targeted and control cells.

The expression of the other two-components of the NALP3 Inflammasome, ASC and NALP3, was also tested. As can be observed in Figure 40, the expression of ASC and NALP3 was not significantly altered upon SFRS3 knockdown. Moreover, 24 hours after challenge a reduced expression of NALP3 is observed in SFRS3 targeted cells, thus suggesting that the increased Caspase-1 activation is not due increased expression of ASC or NALP3.

a) **b)**

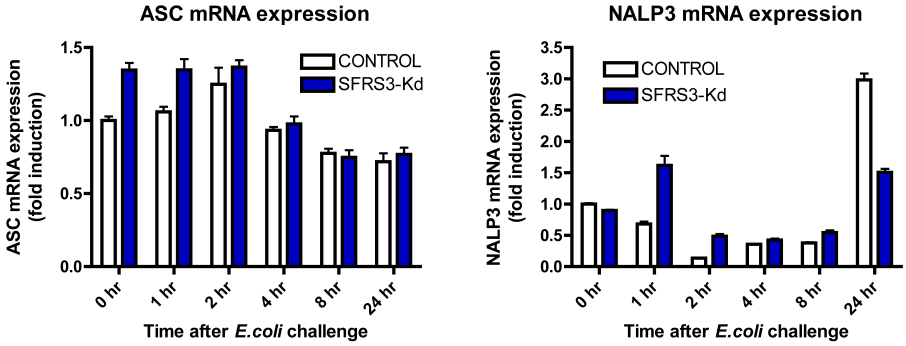


Figure 40 - Kinetics of ASC and NALP3 mRNA expression upon SFRS3 knockdown. THP-1 cells were infected with lentiviral vectors encoding specific shRNAs targeting SFRS1 or SFRS3. Cells were further challenged with *E.coli* for different time points. a) ASC mRNA expression kinetics is not altered upon SFRS3 knockdown. b) NALP3 mRNA expression is not dramatically affected upon SFRS3 knockdown. Small increase can be observed at 1 and 2 hr after *E.coli* challenge. At 24 hr after bacterial challenge, NALP3 expression is reduced in SFRS3 knocked-down cells.

3.2.3.2.2 - The regulation of different Inflammasomes by SFRS3

In the past years, several Inflammasomes have been reported to exist, and to be important in the processing of IL-1β in response to different stimuli (Figure 7, reviewed in *Franchi et al.*, 2009; *Martinon et al.*, 2009). In Chapter 3.1.3.2, we have reported that the NALP3 Inflammasome is the major Inflammasome involved in the generation of IL-1β response to an *E.coli* challenge. However, we cannot rule-out the possibility that by knocking-down SFRS3, we might be activating different Inflammasomes, and consequently an increased Caspase-1 activation.

To answer this question, we performed double knockdown experiments targeting the 3 major Inflammasomes described (NALP3, NALP1 and IPAF), the common adaptor ASC, and at the same time SFRS3. After infection, cells were challenged with *E.coli* for 24 hr. Interleukin-1β secretion was measured in the cell supernatants by ELISA. The results present at Figure 41 show that the increased IL-1β secretion observed upon SFRS3 knockdown, can be partially reverted if a double knockdown with NALP3 or NALP1 is performed. Suggesting that these two Inflammasome platforms might be negatively regulated by SFRS3. In opposition, IPAF does not seem to play a role in the increased IL-1β secretion upon SFRS3 knockdown, since the same levels of IL-1β are secreted when double knockdowns were performed for SFRS3/IPAF. Double knockdowns of SFRS3 and the Inflammasome(s) adaptor ASC, remarkably decreased the IL-1β secretion, therefore confirming the important role of ASC in IL-1β secretion, as was shown previously.

These results thus suggest that the increased IL-1β secretion upon SFRS3 knockdown might be also due to activation of different Inflammasome platforms,

in addition to the increased Caspase-1 expression shown in the previously. However, further experiments are required to confirm this assumption.

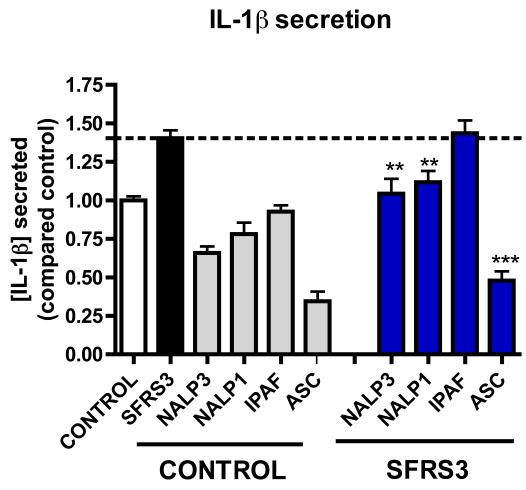


Figure 41 – The role of different Inflammasomes in IL-1β secretion upon SFRS3 knockdown. Several key-components of the different Inflammasomes and SFRS3 were double knockdown in THP-1 cells, either in the presence of Scramble shRNA (Control) or SFRS3. The increased IL-1β secretion observed in the double knockdown Control/SFRS3 situation is reverted if in addition to SFRS3 kd, NALP3 or NALP1 are also knocked-down. Double knockdown of SFRS3/IPAF does not decrease IL-1β secretion as compared to the Control/SFRS3 double knockdown. The double knockdown of SFRS3 and the adaptor protein ASC, shows a remarkable decreased IL-1β secretion after 24 hr *E.coli* challenge. (t-student:**$p<0.005$, ***$p<0.0001$).

3.2.4 - SFRS1 and SFRS3 Regulation upon *E.coli* challenge

As reported previously, ASF/SF2 expression was shown to be downregulated in inflamed muscle and in the presence of a pro-inflammatory cytokine (*Xiong, 2006)*. Thus, these results prompted us to look for the expression of SFRS1 and SFRS3 in our model system, THP-1 cells challenged with *E.coli*.

Concerning the expression of SFRS1, Figure 42-a shows that its mRNA expression is down regulated after *E.coli* challenge, already 1 hr post-challenge. This result seems to be in agreement with the involvement of SFRS1 in Inflammation as described by *Xiong et al.* (*Xiong*, 2006). In opposition, SFRS3 mRNA expression is not remarkably changed along the stimulation. Although, at 24 hr after *E.coli* challenge, it seems to be down-regulated, but not to similar levels as compared with SFRS1 (Figure 42-b).

The expression and activity of several SFs have also been shown to be regulated at post-transcriptional levels (e.g. by miRNAs, *Boutz et al.*, 2007) and at post-translational levels (such as phosphorylation, as described in Chapter 1.2.1.1). Since we were interested in studying the role of SFRS3 in the regulation of IL-1β secretion, in addition to SFRS1, we decided to go further and look for possible SRp20 alterations induced by an *E.coli* stimulus. As can be seen in Figure 42-c, SRp20 expression is down regulated at the protein level after *E.coli* challenge.

In sum, we can conclude that the expression of these two SFs is altered upon *E.coli* challenge. The underlying mechanism that regulates their expression is not yet known, however is now under study.

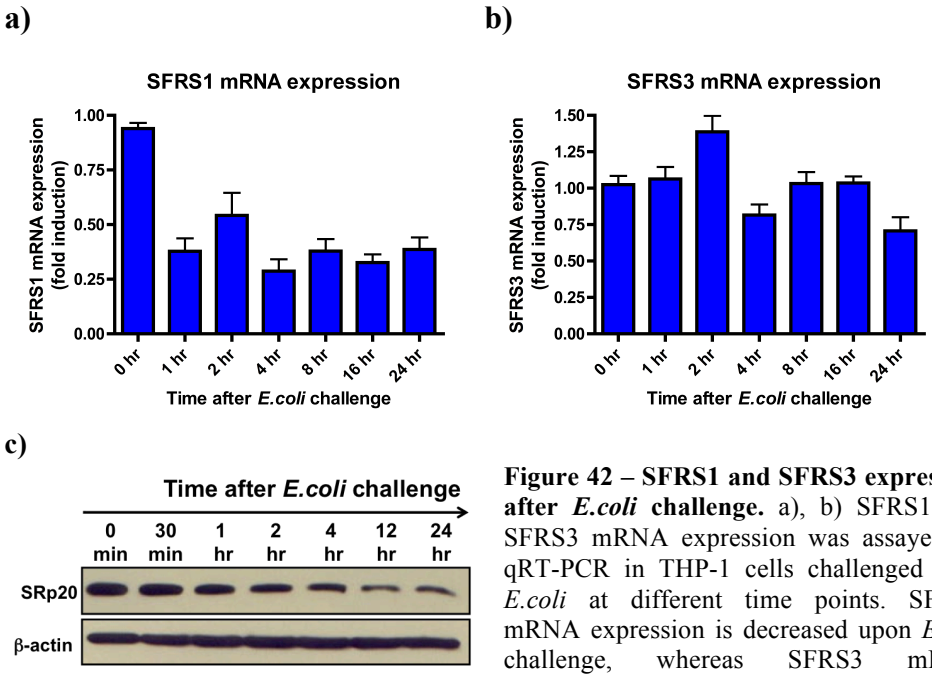


Figure 42 – SFRS1 and SFRS3 expression after *E.coli* challenge. a), b) SFRS1 and SFRS3 mRNA expression was assayed by qRT-PCR in THP-1 cells challenged with *E.coli* at different time points. SFRS1 mRNA expression is decreased upon *E.coli* challenge, whereas SFRS3 mRNA expression is almost unaltered. An exception can be observed at 24 hr post challenge where SFRS3 mRNA expressed levels are lower, although not at the same extent as SFRS1. c) THP-1 cells challenged with *E.coli* show decreases SRp20 expression over time, as detected by Western Blot. β-actin was used as protein loading control.

3.2.5 – Alternatively Spliced Genes involved in the regulation IL-1β secretion

As discussed in Chapter 1.1.3a, numerous isoforms of important genes involved in the IL-1β secretion pathway have been found over time (reviewed in *Leeman et al., 2008; Krieg et al., 2009; Matsushita et al., 2009a; Sahoo et al., 2010)*. However, a lot is still unknown about the true extent of Alternative Splicing events (ASE) that are involved in the regulation of this Inflammatory response. In an attempt to identify possible Splicing events that occur upon *E.coli* challenge, the

Splicing patterns of several genes already described to play a role in IL-1β secretion were tested. In addition to the already identified isoforms, we extended our AS analysis to other putative isoforms and genes that could be involved in the regulation of IL-1β secretion. We have based our search on published data and using a bioinformatic tool, the Exonmine online database (http://www.imm.fm.ul.pt/exonmine/), that predicts the different isoforms each gene may present. According to the different isoforms already published and the putative isoforms predicted by the Exonmine database, we have designed the primers that would allow us to identify the most interesting Splicing patterns of the genes in study (listed at Table 7). Alternative Splicing patterns were then analyzed by RT-PCR followed by agarose gel electrophoresis. However, besides the greater number of genes tested, no conclusive results were obtained in what concerns the possible isoforms induced by an *E.coli* challenge.

Table 7 – List of genes tested for ASE upon *E.coli* challenge of THP-1 cells.

GENE	GENE	GENE	GENE
APAF1	CASP2	MDM2	P2X7
ASC	CASP8	MEFV2	PANX1
BCL-2	CASP9	MKNK2	PANX2
BCL-X	CD45	MyD88	PANX3
BCL10	COP1	NALP1	PTEN
BCL2A1	ICEBERG	NALP1B	RIPK2
BIN1	IL1RN	NALP3	RPS6KB
CARD9	INCA	NLRX1	SERPINB2
CASP1	MALT1	P2X4	SFRS1

Similar experiments were performed in THP-1 cells previously targeted with control, SFRS1 and SFRS3 specific shRNAs. With this approach we attempted to identify possible alterations in the Splicing patterns of these genes, that could be regulated by the two SFs. Moreover, as described in previous

Chapters, knocking-down the expression of the SFs can also affect gene expression, as demonstrated by the increased Caspase-1 mRNA expression upon SFRS3 knockdown. Therefore, we have extended our approach to identify possible alterations at gene expression levels, in addition to the different isoforms. However, once more, no conclusive results were obtained using this approach (data not shown).

Thus, we decided to perform a high-throughput analysis of ASE and differences of Gene Expression (DEG) using the GeneChip® Human Exon 1.0 ST Arrays platform by Affymetrix®.

3.2.5.1 - Exon-array analysis

The GeneChip® Human Exon 1.0 ST Arrays platform by Affymetrix® is designed to have approximately four probes covering each exon, and roughly 40 probes per gene, therefore enabling two complementary levels of analysis, gene expression (differences in gene expression, DEG) and Alternative Splicing events (ASE), The existence of several probes for the same exon enables "exon-level" analysis, therefore allowing to distinguish differentially expressed isoforms of the same gene. In addition, the large number of probes covering the same gene also makes this platform a good method to evaluate the expression levels of a certain gene (http://www.affymetrix.com/products_services/arrays/specific/exon.affx).

In order to perform this analysis, THP-1 cells were infected with lentivirus encoding an shRNA targeting SFRS1 or Control. After puromycin selection, cells were challenged for 4 hr with *E.coli*. Total RNA was extracted and cell supernatants were assayed for IL-1β secretion by ELISA. As can be seen in Figure 43-a, 4 after *E.coli* challenge, SFRS1 knocked-down cells show increased IL-1β

secretion, similar to what was observed 24 hr post-challenge. SFRS1 knockdown was validated at mRNA (Figure 44-b) and protein levels (data not shown).

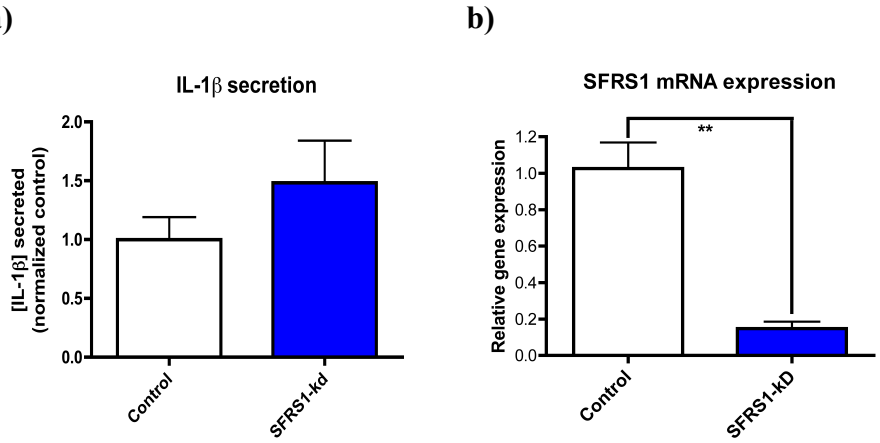


Figure 43 – Validation of SFRS1-kd phenotype in IL-1β secretion. THP-1 cells were infected with lentiviral vectors encoding an shRNA to target SFRS1. a) IL-1β secretion was assayed in the cell supernatants after 4 hr of *E.coli* challenge. The values were normalized against the IL-1β secreted levels measured in control-infected cells. An increase in IL-1β secretion was observed in SFRS1 knocked-down cells. b) The efficiency of SFRS1 knockdown was assayed by qRT-PCR and normalized against the expression of SFRS1 in control infected cells. (t-student: **$p<0.005$).

The RNA extracted was sent to the Genomics Core Facility at The European Molecular Biology Laboratory in Heidelberg, Germany (EMBL) for hybridization in the GeneChip® Human Exon 1.0 ST Arrays (http://www.embl.de/services/core_facilities/genecore/index.html). After the hybridization protocol, the data generated was analyzed in collaboration with *Ana Grosso* at the Unit of Cellular Biology (UBCe) at Instituto de Medicina Molecular (IMM) in Lisbon, Portugal, as described in Chapter 2.2.10. Twelve hybridizations were performed: Control cells stimulated and non-stimulated with *E.coli*, Control-S and Control-NS, respectively; and SFRS1-kd cells stimulated (shSF2-S) and non-stimulated with *E.coli* (shSF2-NS, all in triplicate). Two types of analysis

were performed for the different replicate samples, differences in gene expression (DEG, Chapter 3.2.5.1.1) and in Splicing patterns (ASE, Chapter 3.2.5.1.2).

3.2.5.1.1- Differentially Expressed Genes (DEG)

The Human Exon 1.0 ST Arrays platform allows the identification of DEG due to the high number of probes covering each gene, as mentioned before. Three comparison analyses were performed with the data generated: Control-NS vs Control-S, shSF2-NS vs Control-NS and shSF2-S vs Control-S (Figure 44). The complete list of genes DEG in the different analysis performed can be seen on supplementary data.

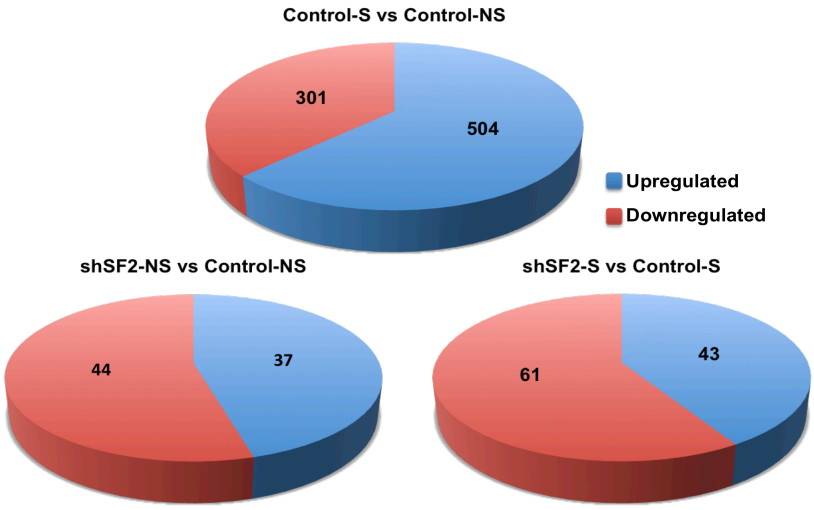
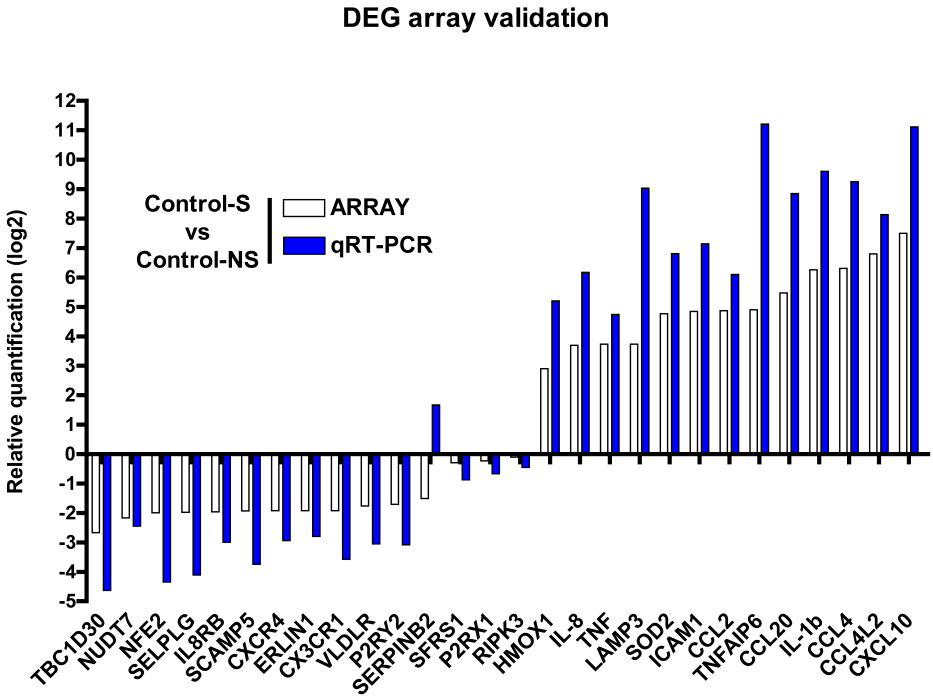


Figure 44– DEG obtained in the different comparison analysis. After bioinformatics analysis of the data generated by the Human Exon 1.0 ST Arrays, the expression of several genes was shown to be changed in the different conditions, either upregulated (in blue) or down regulated (in red).

In order to validate the results obtained by the bioinformatics analysis, qRT-PCR experiments were performed for 33 of the genes found to be differentially expressed in the different comparisons (the validated genes are listed at Table 8). On Figure 45 are shown the results obtained in the qRT-PCR experiments (in blue) in comparison to the fold inductions determined by the array analysis (in white). The results obtained in the Control-S vs Control-NS analysis are represented in Figure 45-a, whether the shSF2-Ns vs Control-NS analysis is shown in Figure 45-b. The primers used for the validations are listed in Table S5. Among the genes tested only SERPINB2 was not validated (as can be seen in Figure 45). The rate of validation was 96.9%.

a)



b)

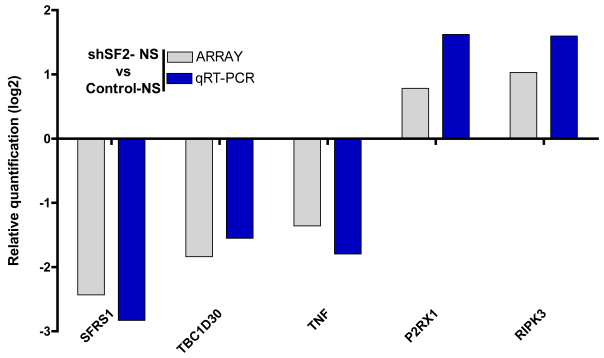


Figure 45 – Validation of the DEG obtained by bioinformatic analysis of the array data. qRT-PCR experiments were performed to detect differences at gene expression levels of some differently expressed candidates, obtained by bioinformatic analysis of the Exon arrays. In the Figure are represented the fold inductions predicted by the array (in white) and the fold inductions obtained in qRT-PCR analysis (in blue). a) Control-S vs Control-NS analysis validation-All the genes show a similar profile in the qRT-PCR analysis when compared to the predicted by the array, with the exception of the SERPINB2 gene, therefore not validated. b) shSF2-Ns vs Control-Ns analysis validation-All the genes analyzed were validated.

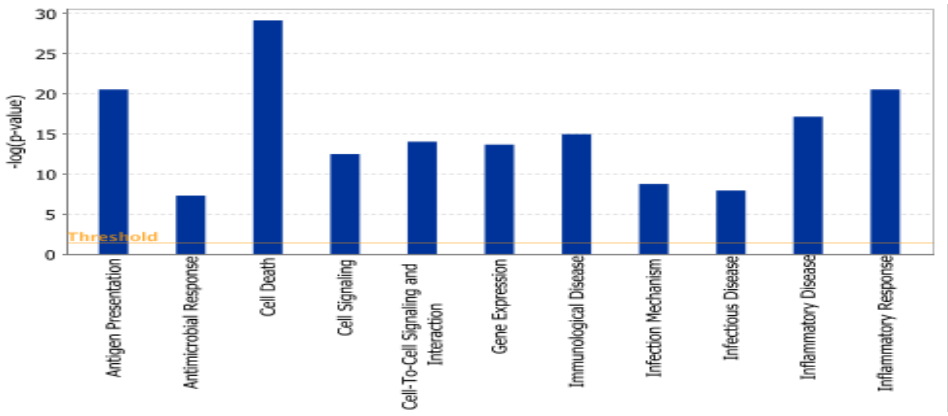
Table 8 – List of DEG genes validated by qRT-PCR in the different analysis.

Control-S vs Control-NS	Control-S vs Control-NS	shSF2-NS vs Control-NS
CCL2	NFE2	P2RX1
CCL20	NUDT7	RIPK3
CCL4	P2RX1	SFRS1
CCL4L2	P2RY2	TBC1D30
CX3CR1	RIPK3	TNF
CXCL10	SCAMP5	
CXCR4	SELPLG	
ERLIN1	SFRS1	
HMOX1	SOD2	
ICAM1	TBC1D30	
IL-1β	TNF	
IL-8	TNFAIP6	
IL8RB	VLDLR	
LAMP3		

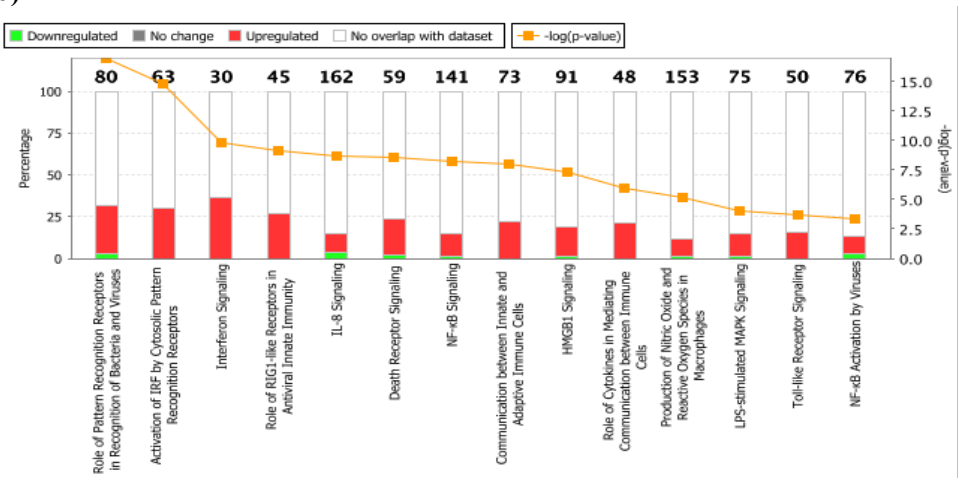
In agreement to what was already presented previously, IL-1β and IL-8 mRNA expression is induced after *E.coli* challenge. As can be seen in Figure 45-a, SFRS1 mRNA expression is down-regulated after an *E.coli* challenge, further proving the results presented before.

A preliminary bioinformatic analysis was performed using the Ingenuity® Pathway analysis software (INGENUITY® Systems) in order to evaluate the major pathways and cellular functions that are either activated or inhibited in the different conditions (*E.coli* challenge or SFRS1 knockdown). As can be seen in Figure 46-a several cellular functions (such as Cell death and Inflammatory response) and pathways (Figure 46-b) are "altered" when challenging THP-1 cells with *E.coli*. Among the pathways induced, is the NF-kB pathway, which was already described to be important, either for the production of IL-1β and in the Inflammatory response in general, therefore validating this approach. Additionally, several genes in the NF-kB pathway are either up- (in red) or down regulated (in green), as can be seen in Figure 46-c. This preliminary approach will be extended in the future to the other analysis in an attempt to find important genes which expression is altered that could have an impact in the regulation of IL-1β secretion after an *E.coli* challenge.

a)



b)


Downregulated
No change
Upregulated
No overlap with dataset
-log(p-value)
Percentage
80 63 30 45 162 59 141 73 91 48 153 75 50 76
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
Activation of IRF by Cytosolic Pattern Recognition Receptors
Interferon Signaling
Role of RIG1-like Receptors in Antiviral Innate Immunity
IL-8 Signaling
Death Receptor Signaling
NF-κB Signaling
Communication between Innate and Adaptive Immune Cells
HMGB1 Signaling
Role of Cytokines in Mediating Communication between Immune Cells
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
LPS-stimulated MAPK Signaling
Toll-like Receptor Signaling
NF-κB Activation by Viruses

c)

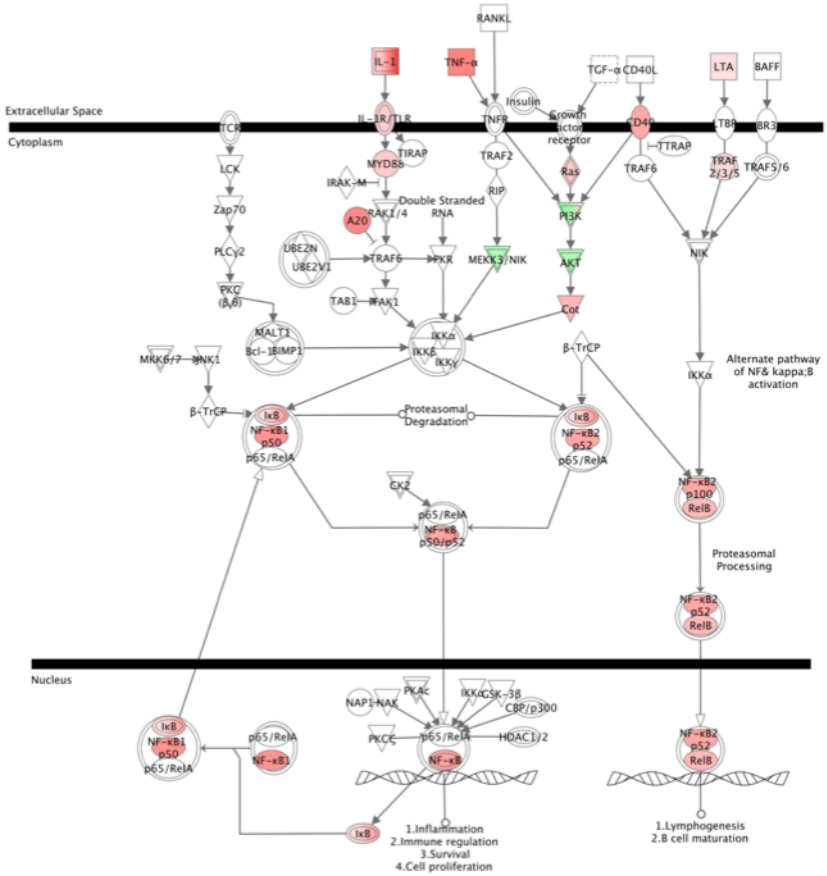

RANKL
IL-1
TNF-α
TGF-α
CD40L
LTA
BAFF
Extracellular Space
Cytoplasm
Insulin
TCR
IL-1R/TLR
TNFR
Growth factor receptor
CD40
TBR
BR3
TRAP
TRAF2
TRAF6
TRAF 2/3/5
TRAF5/6
MYD88
IRAK-M
RIP
Ras
PI3K
Double Stranded RNA
RAK1/4
A20
LCK
Zap70
PLCγ2
UBE2N
UBE2V1
TRAF6
PKR
MEKK3/NIK
AKT
NIK
PKC (δ,θ)
TAB1
TAK1
Cot
MALT1
Bcl-10
IBIMP1
IKKα
IKKβ
IKKγ
MKK6/7
JNK1
β-TrCP
Alternate pathway of NFκ kappa;B activation
IκB
NF-κB1 p50
p65/RelA
Proteasomal Degradation
NF-κB2 p52
CK2
NF-κB p50/p52
NF-κB2 p100 RelB
Proteasomal Processing
Nucleus
NAP1-NAK
PKAc
IKKα
GSK-3β
CBP/p300
PKCε
HDAC1/2
1.Inflammation
2.Immune regulation
3.Survival
4.Cell proliferation
1.Lymphogenesis
2.B cell maturation

Figure 46 – Ingenuity Pathway analysis of the DEG obtained in the Control-S vs Control-NS Exon array analysis. a,b) Several cellular function and pathways are induced after 4 hr *E.coli* challenge. In b is represented the percentage of the known genes in different pathways that are either up- or down regulated. Among the different pathways altered is the NF-KB pathway. c) Close look to the genes in the NF-KB pathway that are altered after 4 hr *E.coli* challenge. Up and down regulated genes are represented in red and green, respectively.

3.2.5.1.2 - Alternative Splicing Events (ASE)

In an attempt to identify possible changes in the Splicing pattern of different genes upon *E.coli* challenge and/or SFRS1 knockdown by shRNA, several comparison analysis were performed with the data originated by the Human Exon 1.0 ST Arrays for each sample, namely Control-S vs Control-NS, shSF2-NS vs Control-NS and shSF2-S vs Control-S, as described previously for the case of DEG. The complete list of ASE found to occur in the different analysis performed is present on Chapter 7. In order to validate the results, PCR analysis were performed using specific primers to detect the ASE events predicted by the Exon array. The primers (listed on Table S4) were designed in accordance to the different isoforms predicted by the array and by different isoforms of the same gene already listed at the Exonmine online database (http://www.imm.fm.ul.pt/exonmine/). An example of a validated and a non-validated ASE is present on Figure 47, for OS9 and SPPL3 genes, respectively. The list of ASE tested is present at Table 9. All the validated ASE are depicted in bold, therefore, the rate of validation was 78% for the Control-S vs Control-NS, and 67% for the shSF2-NS vs Control-NS.

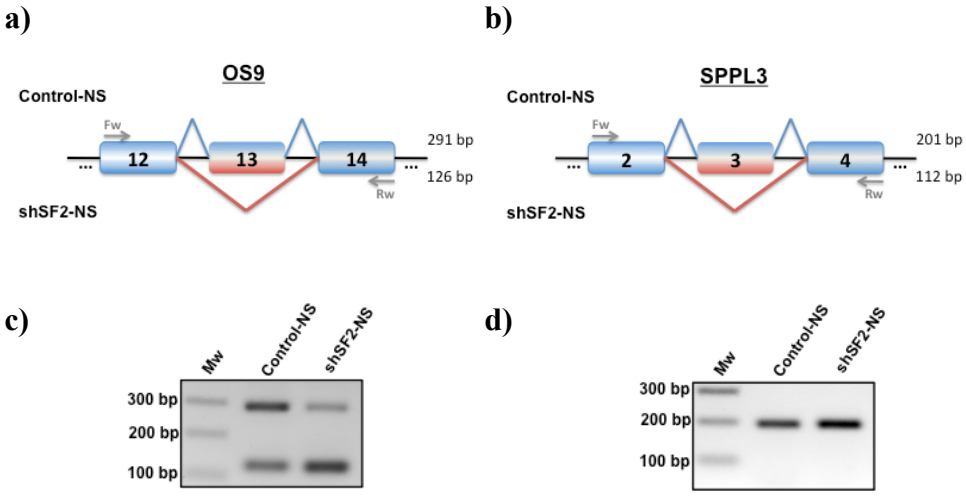


Figure 47 – ASE validation. ASE predicted by the Exon array analysis were validated by PCR. a,b) Schematic representation of the ASE predicted by the array for 2 of the genes, OS9 and SPPL3, respectively. The numbers correspond to the number of the exon listed on the Exonmine database. The primers and the respective annealing sites are represented in grey as forward (Fw) and reverse primers (Rw). In the case of OS9, exon 13 is excluded upon ASF/SF2 knockdown, generating a shorter isoform of 126 bp when compared to the control situation, 291 bp. For SPPL3, exon 3 is excluded upon ASF/SF2 knockdown, originating a 112 bp isoform. c,d) PCR validation of the array predictions. c) After ASF/SF2 knockdown, there is an increase of the shorter band (126 bp) as predicted by the array, therefore validating the ASE. In the case of SPPL3 (d), the prediction by the array suggested that a shorter isoform of 112 bp would be amplified in cells targeted to ASF/SF2 knockdown. No differences in isoforms were seen, therefore not validating this ASE.

Table 9 – List of tested and validated (in bold) ASE in the different analysis.

Control-S vs Control-NS	shSF2-NS vs Control-NS
ATP2B4	**ALAS1**
CD44	**IRF7**
EIF4H	**OS9**
IL-32	PVRL2
RET	SPPL3
RGS8	**STAT5A**
SHD	
STAT5A	
TNFAIP2	

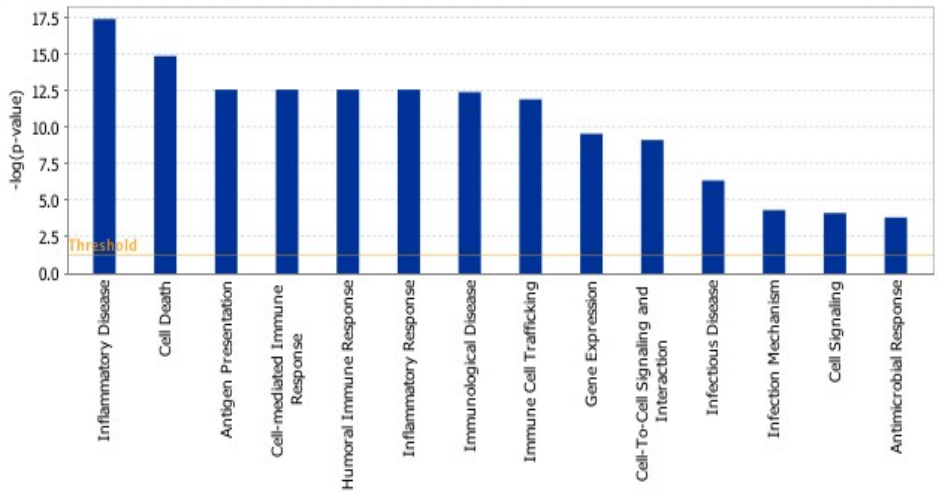
In a similar way to what was done in the previous Chapter for the DEG, a preliminary bioinformatic analysis using Ingenuity® software was performed for the ASE. As can be seen in Figure 48-a several immune functions are altered at an "Alternative Splicing" level, for example the Inflammatory response. Several signaling pathways are also altered, such as the expected NF-kB pathway (Figure 48-b). Numerous genes in the NF-kB pathway were already reported to be regulated by AS and to play a role in the regulation of this pathway (reviewed in *Leeman et al.*, 2008). Furthermore, some of the isoforms identified in this pathway (e.g. MyD88s) were already reported to be induced by an Inflammatory stimulus, such as LPS (*Janssens et al.*, 2002; *Janssens*, 2003), thus validating our approach. In Figure 48-c are represented the ASE in the NF-KB pathway found by the Exon array analysis. In red and green are represented exon inclusion and exclusion events, respectively.

We decided to intersect the list of genes that undergo AS listed by *Leeman* and *Gilmore* (*Leeman et al.*, 2008) and the events collected by our Exon Array analysis (Control-S vs Control-NS). Doing this, we observed that 36% of the genes listed in the report were among the ASE detected by the array (represented by asterisk in Figure 48-c and shown in Table 10).

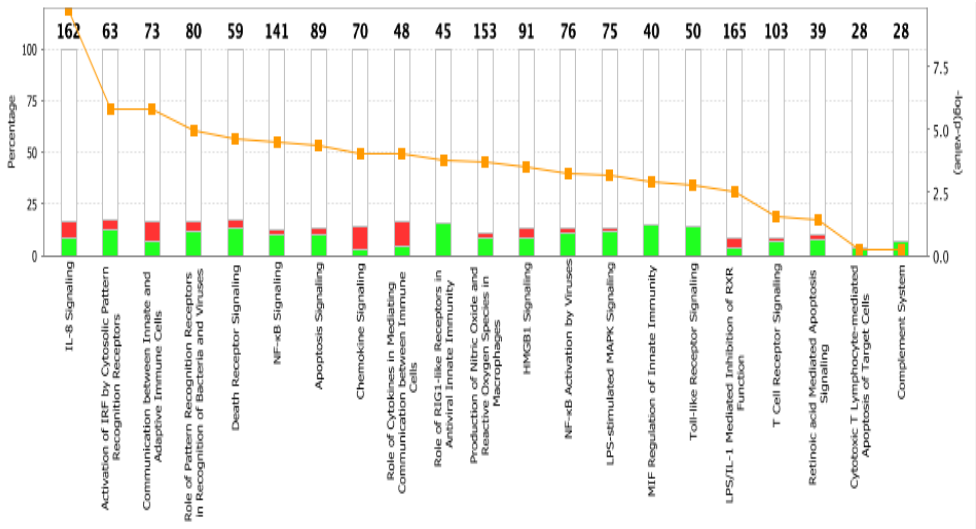
In addition, a similar approach was performed to determine which ASE predicted by the Exon array were already described in TLR signaling cascade by *Wells et al.* (*Wells et al., 2006*). As presented in Table 10, several ASE predicted in our analysis were already reported, such as NFKB1 or NFKB2, among others (approximately 20% of the already described ASE were found in our analysis).

We therefore hypothesize that this approach can be extended in the future to find new important genes in the Inflammatory response that are regulated by AS.

a)


-log(p-value)
17.5
15.0
12.5
10.0
7.5
5.0
2.5
0.0
Threshold
Inflammatory Disease
Cell Death
Antigen Presentation
Cell-mediated Immune Response
Humoral Immune Response
Inflammatory Response
Immunological Disease
Immune Cell Trafficking
Gene Expression
Cell-To-Cell Signaling and Interaction
Infectious Disease
Infection Mechanism
Cell Signaling
Antimicrobial Response

b)


Percentage
-log(p-value)
162 63 73 80 59 141 89 70 48 45 153 91 76 75 40 50 165 103 39 28 28
IL-8 Signaling
Activation of IRF by Cytosolic Pattern Recognition Receptors
Communication between Innate and Adaptive Immune Cells
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
Death Receptor Signaling
NF-κB Signaling
Apoptosis Signaling
Chemokine Signaling
Role of Cytokines in Mediating Communication between Immune Cells
Role of RIG1-like Receptors in Antiviral Innate Immunity
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
HMGB1 Signaling
NF-κB Activation by Viruses
LPS-stimulated MAPK Signaling
MIF Regulation of Innate Immunity
Toll-like Receptor Signaling
LPS/IL-1 Mediated Inhibition of RXR Function
T Cell Receptor Signaling
Retinoic acid Mediated Apoptosis Signaling
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells
Complement System

c)

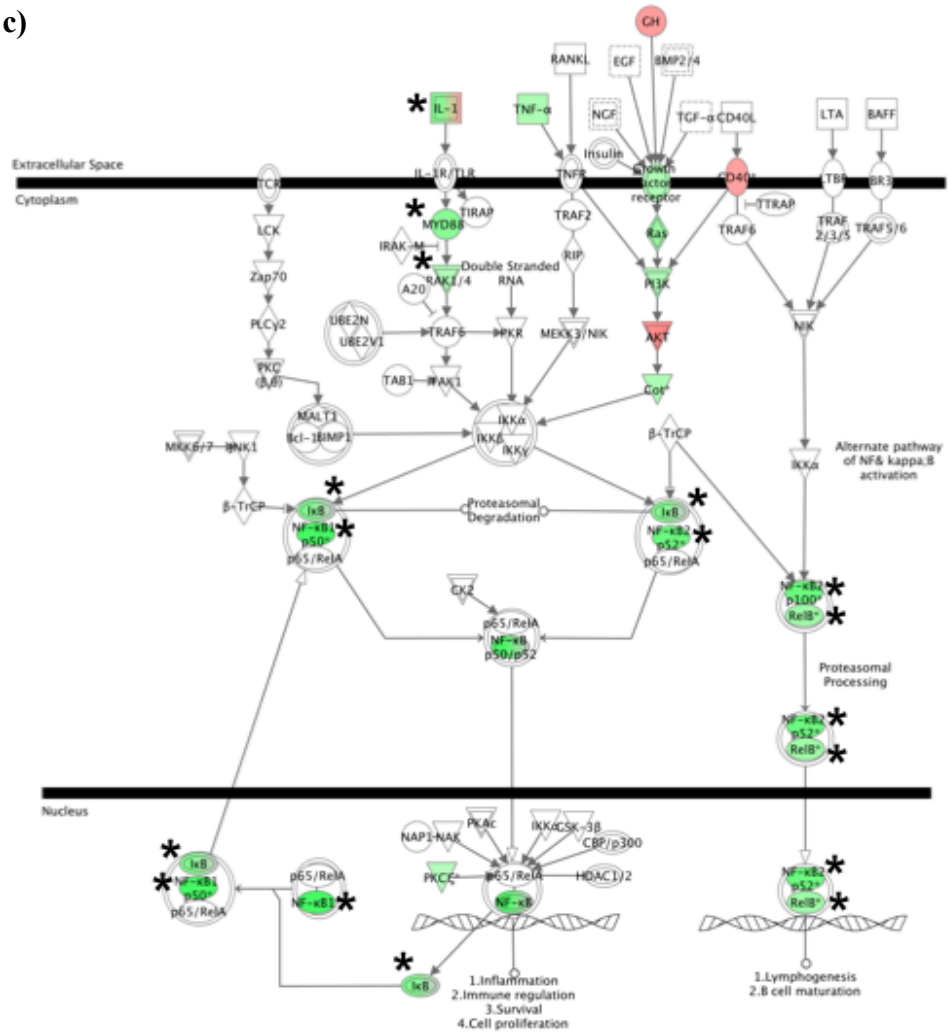


Figure 48 – Ingenuity Pathway analysis of the ASE obtained in the Control-S vs Control-NS Exon array analysis. a,b) Several cellular functions and pathways are altered at AS level after *E.coli* challenge. In b) is represented the percentage of the genes in the different pathways that undergo AS by exon array analysis prediction, c) Close look to the genes in the NF-KB pathway that undergo AS after *E.coli* challenge. Exon exclusion events in green, and exon inclusion events in red. The genes marked with asterisk (*) are the genes that were already reported, by *Leeman and Gilmore* (*Leeman et al., 2008*), to undergo AS in the NF-kB pathway.

A similar approach was also performed, by crossing the events already described in the two mentioned reports and the ASE predicted by the remaining analysis (shSF2-NS vs Control-NS and shSF2-S vs Control-S), however, as expected showing a decreased level of intersection (Table 10).

Table 10 - Intersection of the ASE predicted by the different Exon Array analysis and ASE already reported in similar studies.

Comparison	Genes in common with *Leeman* report (*Leeman et al.*, 2008)	Genes in common with *Wells* report (*Wells et al.*, 2006)
Control-S vs Control-NS	IL1RN, IRAK1, IRAK2, MyD88, NFKB1, NFKB2, NFKBIZ, REL, RELB	CCL5, CXCL10, IL12B, NFKB1, NFKB2, NFKBIA, RAC2, TNF
shSF2-NS vs Control-NS	NFKBIB	
shSF2-S vs Control-S	IRAK1, REL, TLR4,	RAC2

Recently, seminal work from different groups have been focused in the identification of the genes that could be regulated by different SFs, such as the ASF/SF2. In 2008, *Sanford et al*. identified, by Cross-Linking Immunoprecipitation (CLIP), 326 binding sites for SF2/ASF in RNA transcripts from 180 protein coding genes (*Sanford et al.*, 2008). A more recent paper, also from *Sanford et al*., using a Cross-linking Immunoprecipitation and high-throughput sequencing technique (CLIP-seq), allowed the identification of numerous binding sites for the ASF/SF2 in the transcriptome of cultured embryonic kidney cells (*Sanford et al.*, 2009). Therefore, due to the available CLIP-seq data for ASF/SF2, we decided to perform a bioinformatic approach to check which of the ASE that were found to occur by the Exon array analysis, would be present in those set of genes already published by *Sanford. et al.*

(*Sanford et al.*, 2009), listed in Tables 11 and S18. Several of the genes identified by the ASF/SF2 CLIP-seq assay, were predicted by the Exon array analysis to have altered Splicing events once targeting this SF by RNAi. We are at the moment choosing the most interesting candidates to play a putative role in the regulation of the IL-1β secretion for further studies.

Table 11 – List of the common genes identified in the ASF/SF2 CLIP-seq analysis (*Sanford et al.*, 2009) and the ASE observed in the shSF2-NS vs Control-NS analysis.

Common ASE of shSF2-S vs Control-S analysis and ASF/SF2 CLIP-seq (*Sanford et al.*, 2009)			
ABCE1	HDLBP	PHPT1	SLC6A8
ACADVL	HGS	PI4KB	SMPD4
ADCK4	HMGA1	PNPO	SOLH
ADNP	HNRNPD	POLR1D	SPATA5L1
AHSA1	KIAA0515	POMP	SRP9
APLP2	KIAA1191	PPIE	SRRM2
ARHGAP17	LEPRE1	PPIH	STAU1
ARL17	MYL6	RAB5C	STK40
C7orf27	NDUFB8	RAD54L	TAP2
CDC2L5	NMT1	RBM3	TMEM109
CDC42SE1	NOMO2	RPL28	TRIM37
CHAF1A	OS9	RPL35	UBAP2L
COX11	PBX4	RPN2	UCHL1
FAM128B	PCBP2	RPS21	YWHAB
GEMIN7	PEA15	RRP1	ZNF202
H2AFX	PFKP	RRP12	

Chapter 4 - Discussion

"Professionals know that they have to produce theory after theory before they are likely to hit the jackpot."

Francis Crick

The Inflammation process is thought to have been first reported by *Celsus*, in the first century A.D., with the description of the four signs of Inflammation (reviewed in *Rocha e Silva, 1978; Rocha e Silva, 1994; Medzhitov, 2010)*. Along the years, several other descriptions have been made, however, perhaps the biggest advances in the knowledge of this Immune Response, started to arise in the nineteenth century by *Virchow*, *Cohnheim, Weigert, Metchnikoff,* among others (*Virchow*, 1871; *Cohnhein*, 1873; *Weigert*, 1889; *Metchnikoff*, 1892; *Rocha e Silva*, 1978)

"*He (Metchnikoff) put forward the principle that Inflammation was a reaction of mesodermal cells against an external agent and that Inflammation was a curative reaction*." (*Turk*, 1991)

Since then, several important discoveries have outlined the importance of a tight regulation of Inflammation in the outcome of a microbial infection and several other inflammatory conditions where the microbe presence is not mandatory (reviewed in *Medzhitov*, 2008). Along the past years, several new "highways" of research have been "opened", namely after the discovery of the release of pro-inflammatory mediators by immune cells (such as cytokines, *Menkin*, 1944), the concept of Immune PRRs by Janeway's (*Janeway*, 1989) and more recently with the discovery of the Inflammasome (*Martinon et al., 2002*).

The IL-1β was one of the first cytokines to be identified and represents one of the most important mediators of Inflammation and host responses to infection (reviewed in *Dinarello*, 2004). The strong connection between misregulation of IL-1β release and the appearance of inflammatory diseases, made this cytokine one of the "hotspots" of intensive research (reviewed in *O'Neill*, 2008). A large number

of diseases have been reported to be caused by a misregulation of this inflammatory response, for example, several mutations in key-components of this response have been linked to the appearance of such diseases, such as the case of Familial Cold Autoinflammatory Syndrome or the Muckle-Wells Syndrome that are linked with mutations in NALP3 (*Ogura et al.*, 2001; *Maeda et al.*, 2005; *McGonagle et al.*, 2007; *Church et al.*, 2008; *Martinon et al.*, 2009). Nowadays, certain diseases are already being treated successfully using IL-1β receptor antagonists, such as Anankira, or other methods to decrease IL-1β concentrations, for example using monoclonal anti-IL-1β antibodies (*Fitzgerald et al.*, 2005; *Kalliolias et al.*, 2008; *Lequerre et al.*, 2008; *Martinon et al.*, 2009). However, despite several years of intensive research, the mechanisms involved in the regulation of IL-1β secretion are not fully known, therefore, still of extreme importance to be studied.

The discovery of the Inflammasome, and other studies focused on the regulation of IL-1β secretion, have been performed using the THP-1 monocytic cell line (*Martinon et al.*, 2002; *Sarkar et al.*, 2006; *Burckstummer et al.*, 2009; *Fernandes-Alnemri et al.*, 2009). In some of the studies researchers differentiated the monocytic cells into macrophages by different methods, such as using phorbol 12-myristate 13-acetate (PMA, *Martinon et al.*, 2002; *Dostert et al.*, 2008; *Vinzing et al.*, 2008). We have decided to perform our studies in non-macrophage differentiated THP-1 cells. The usage of PMA has been reported by *DeCoursey et al.* (*DeCoursey et al.*, 1996) to modify the expression of several K^+ ion channels in THP-1 cells. The already reported involvement of K^+ and K^+ channels in Inflammasome activation (*Pétrilli et al.*, 2007) and consequently in IL-1β release (reviewed in *Ferrari et al.*, 2006), strongly suggested us to use a more homogeneous population of undifferentiated cells to perform our high-throughput analysis, where little cell-to-cell changes that would occur upon the differentiation

step would increase the "noise" obtained in the assays. Thus, we performed pilot assays to verify if the non-macrophage differentiated THP-1 cell line would be a good model to study the inflammatory response to an *E.coli* challenge. After bacterial stimulation, as early as 1 hour post *E.coli* challenge, induction of IL-1β expression was detected in THP-1 cells. The active and secreted form of IL-1β was detected in THP-1 supernatants already 2 hr post-challenge and kept raising till 48 hours after bacterial stimulus. The results obtained show that differentiation of THP-1 cells into a macrophage like cells is not mandatory to study IL-1β secretion upon *E.coli* challenge.

A number of studies report different activation requirements by monocytes or macrophages, such as the usage of extracellular ATP in the case of macrophages studies, in order to achieve maximum IL-1β secretion (*Walev et al.*, 1995; *Kahlenberg et al.*, 2004; *Pelegrin et al.*, 2006; *Duncan et al.*, 2007; *Netea et al.*, 2008). Exogenously added ATP is thought to induce stronger IL-1β secretion by decreasing intracellular potassium levels, in a P2X7 receptor dependant manner (*Perregaux et al.*, 1994; *Ferrari et al.*, 1997; *Pétrilli et al.*, 2007; *Solle et al.*, 2001). As mentioned previously, we used THP-1 cells in a monocytic cell state, therefore we decided to test the necessity of adding extracellular ATP and the role of the P2X7 receptor in our assays. We observed that exogenously added ATP induced higher IL-1β secretion, although not statistically significant. Consequently, we decided not to use exogenously added ATP in our assays. In opposition, cells that were treated with a known P2X7 inhibitor, KN-62 (*Baraldi et al. 2003; Friedle et al., 2010*), showed a remarkably decrease in IL-1β secretion, hence showing the importance of this receptor in the release of this cytokine by THP-1 cells. So far, the only known P2X7 receptor agonist is ATP, therefore we hypothesize that THP-1 cells do need ATP for complete IL-1β release, still, exogenously ATP addition is not required for its release upon *E.coli* challenge. These results are in agreement with a previous study by *Piccini et al.*

demonstrating that ATP can be released by monocytes upon different PAMPs stimulation, therefore inducing IL-1β secretion in an autocrine manner (*Piccini et al.*, 2008; *Orellano et al.*, 2009).

As mentioned in Chapter 1, processing of pro-IL-1β into its mature and active form is necessary to its secretion (reviewed in *Martinon et al.*, 2009). Numerous reports show that the processing step can be mediated by different proteases (reviewed in *Netea et al.*, 2010). However, it is generally accepted that it is mainly Caspase-1 processing dependant (reviewed in *Lamkanfi et al.*, 2009; *Martinon et al.*, 2009; *Netea et al.*, 2010). We performed a number of assays to confirm the Caspase-1 dependency of IL-1β secretion after challenging THP-1 cells with *E.coli*. Interleukin-1β secretion is diminished either by decreasing the expression of Caspase-1 by RNAi or by blocking its activation with known inhibitors, confirming the necessity of Caspase-1 processing of IL-1β in order to be released. In addition, the expression of Caspase-1 and its activity after *E.coli* challenge was also evaluated. Both, expression and activity were increased after the bacterial challenge. The sum of these results confirm that after an *E.coli* challenge, THP-1 cells secrete IL-1β in a Caspase-1 processing dependant manner, as already described previously by other studies (reviewed in *Lamkanfi et al.*, 2009; *Martinon et al.*, 2009; *Netea et al.*, 2010).

Caspase-1, as IL-1β, is synthesized in an inactive form that needs further processing in order to be active. This processing step is mediated by the Inflammasome complex (*Martinon et al.*, 2002). Since the discovery of the NALP1 Inflammasome (*Martinon et al.*, 2002), several years of intensive research unveil the existence of other Inflammasome complexes, such as the NALP3, IPAF and more recently the AIM2 Inflammasomes (reviewed in *Martinon et al., 2009; Stutz et al., 2009; Latz, 2010; Schroder et al., 2010a*). The different Inflammasomes were shown to assemble in response to several stimuli, such as PAMPS or

DAMPs, leading to the activation of Caspase-1. In an attempt to identify the most important Inflammasome involved in IL-1β secretion upon *E.coli* challenge, we performed an RNAi-based screen targeting all NLRs described so far and CARD-containing proteins. Knocking-down NALP3 and ASC showed the highest impact in IL-1β secretion after 24 hr *E.coli* challenge. Since these two proteins are the "key-components" of the NALP3 Inflammasome, in addition to Caspase-1 (reviewed in *Ogura et al.*, 2006; *Martinon et al.*, 2009), we concluded that the NALP3 Inflammasome is the major Inflammasome involved in IL-1β secretion upon *E.coli* challenge of THP-1 cells. A similar approach was also successfully performed in another study by our group, in collaboration with the group of Prof. Elsa Anes at the "Faculdade de Farmácia da Universidade de Lisboa", where we identified the components of the *Mycobacterium tuberculosis* (*M.tb*) that lead to higher Caspase-1 activation and IL-1β secretion, upon infection of THP-1 cells. In that study, we have also identified the NALP3 Inflammasome as the major Inflammasome activated during an *M.tb* infection (*Mishra et al.*, 2010). The NALP3 Inflammasome is the most studied Inflammasome, as can be clearly seen by the high number of PAMPS and DAMPS already identified to be involved in its activation. Several mechanisms of NALP3 Inflammasome activation and regulation have been proposed, however, no unifying theory is yet known and accepted (reviewed in *Schroder et al.*, 2010b; *Tschopp et al.*, 2010). The appearance of certain disease states have been linked with Inflammasome disregulation (reviewed in *Martinon et al.*, 2009). As an example, mutations in NALP3 are associated with the appearance of the Hereditary Periodic Fevers, such as Muckle-Wells Syndrome or Familial Cold Autoinflammatory Syndrome (*Hoffman et al.*, 2001; *Stutz et al.*, 2009). Mutations in other Inflammasomes and molecules involved in the IL-1β secretion pathway have also been described to be involved in disease generation (reviewed in *McGonagle et al.*, 2007; *Church et al.*, 2008; *Martinon et al.*, 2009; *Stutz et al.*, 2009). Consequently, it urges the

identification of the underlying mechanisms and regulatory elements involved in the regulation of IL-1β secretion.

A large number of immune relevant genes, either in the Adaptative or Innate Immune Systems, have been shown to be regulated by AS (reviewed in *Lynch*, 2004; *Litman et al.*, 2007; *Mourich et al., 2009*). Several studies have already reported the Splicing of some genes in the NF-kB pathway, such as MyD88 (*Janssens et al.*, 2002), IRAK1 (*Jensen et al.*, 2001), TAB1 (*Ge et al.*, 2003), among others (reviewed in *Leeman et al.*, 2008; *Sahoo et al.*, 2010), therefore regulating this pathway, IL-1β secretion and an inflammatory response in general. Taken this into account, we decided to study the impact of AS in the regulation of Inflammation. In particular, we focused in the identification of Splicing related genes (SF) that play a role in the secretion of IL-1β, after an *E.coli* challenge. To approach that question, we performed an RNAi-based screen targeting 425 SF. After screening validation, 19 genes were considered candidates to play a role in the regulation of IL-1β secretion after 24 hr *E.coli* challenge, 12 as positive regulator and 7 as negative regulator candidates. Among the candidate genes, two of the most studied SR proteins were identified, SFRS1 and SFRS3 (reviewed in *Long et al.*, 2009). SFRS1, which encodes ASF/SF2 protein, was indeed the first SF to be identified by *Krainer et al*. (*Krainer et al.*, 1990a) and has been shown to play a role in different cellular processes besides Splicing, such as RNA transcription and protein translation (reviewed in *Sanford et al.*, 2008; *Zhong et al.*, 2009). In the past years, ASF/SF2 and SRp20 (protein encoded by SFRS3) have been implicated in several diseases such as cancer (*Stickeler et al.*, 1999; *Fischer et al.*, 2004; *Karni et al.*, 2007), AIDS (*Asang et al.*, 2008) and more recently SFRS1 has been implicated in Inflammation (*Xiong*, 2006). Therefore, we decided to focus our work in trying to address the role of ASF/SF2 and SRp20 in the regulation of IL-1β secretion.

In order to validate the results obtained by the RNAi based screening, we have decided to overexpress these two SFs and check the impact in IL-1β secretion. Overexpressing both SFRS1 and SFRS3 lead to decreased IL-1β secretion after 24 hr *E.coli* challenge, in opposition to what was verified upon knockdown of these two SF. In addition, we also cloned and overexpressed IL-1β and IL-18 (Figures S1 and S2), in an attempt to rule out indirect effects in IL-1β secretion governed by increased cytosolic total protein concentration. Interleukin-18 have also been shown to be processed by the Inflammasome, in order to be secreted (*Gracie*, 2003; *Martinon et al.*, 2009) . Moreover, unpublished results by our group show that THP-1 cells do not highly express IL-18 even after an *E.coli* challenge, thus, overexpressing IL-18 in THP-1 cells can be used to perform other experiments in the future, regarding Inflammasome activation. Overexpressing IL-1β, lead to increased secretion of this cytokine, whereas IL-18 overexpression did not show any impact in IL-1β secretion (Figure S3). Accordingly, these results confirmed the previous data obtained by the RNAi based screen, showing the negative role of these two SFs in the regulation of IL-1β secretion.

The increased correlation between Splicing defects and disease focused the attention of pharmaceutical companies and researchers to the identification of drugs that would modulate certain cellular responses by targeting some key Splicing components, such as the case of these two SFs. A study performed by *Stoilov et al.* has identified Digoxin as a modulator of SRp20 dependent Splicing, by decreasing its protein levels (*Stoilov et al.*, 2008). Other study, performed by *Keriel et al.* has identified several drugs capable of inhibiting ASF/SF2 dependant Splicing of retroviral proteins (*Keriel et al.*, 2009). We have then decided to use these drugs to confirm the role of these two SFs in the outcome of an inflammatory response elicited by *E.coli* challenge. Targeting ASF/SF2 by either BO-3 or BO-26 drugs (kindly provided by Dr. *Jamal Tazi*), elicited a higher IL-1β secretion after 24 hr *E.coli* challenge, when compared to control treated cells. Similar results were

obtained upon decreasing SRp20 expression using Digoxin. The results obtained with these assays further complement and confirm the results obtained in the screen and overexpression experiments, as unveiling a role of SFRS1 and SFRS3 as negative regulators of IL-1β secretion. Moreover, the notion of modulating IL-1β secretion and an inflammatory response using drugs targeting specific SFs can be of extreme importance in the future. An increasing number of researchers are now focused in the identification of compounds capable of modulating the activity of certain SF, and some already proved to be successful in certain disease conditions (*Keriel et al., 2009*). Perhaps, in the future, similar set of studies can be performed targeting the identified SFs to control severe inflammatory diseases.

Interleukin-1β secretion is normally defined as a "two-step" mechanism that requires the Production, mainly NF-kB dependant, and the Processing by the Inflammasome (reviewed in *Creagh et al.*, 2006; *Lee et al.*, 2007; *Dinarello*, 2009; *Martinon et al.*, 2009). In order to evaluate the role of the two SFs candidates ASF/SF2 and SRp20 in the two-steps necessary for IL-1β secretion, several experiments were performed. In addition to IL-1β, we decided to evaluate the production of IL-8 by THP-1 cells after an *E.coli* challenge. Interleukin-8 is also an important pro-inflammatory mediator (reviewed in *Miller et al.*, 1992; *Feghali et al.*, 1997). Upon stimulation, IL-8, as IL-1β, is mainly produced in an NF-kB dependant manner, however, in opposition to IL-1β, it is produced in its active form where no further processing steps are required in order to be secreted (*Standiford et al.*, 1990; reviewed in *Remick*, 2005). The production of the two inflammatory cytokines, IL-1β and IL-8, was previously shown to be induced upon *E.coli* stimuli. Therefore, to evaluate if the increased IL-1β secretion upon SFRS1 or SFRS3 knockdown was due to increased cytokine expression, we have measured IL-1β and IL-8 mRNA expression upon targeting the two SF by RNAi. Increased mRNA expression of both inflammatory cytokines was detected upon

SFRS1 or SFRS3 knockdown, when compared to control cells. Moreover, the expression levels of both cytokines, IL-1β and IL-8, were highly increased in cells targeted for SFRS3, when compared to SFRS1 knockdown, correlating with the even higher cytokine secretion levels observed in SFRS3 knockdown cells (Figures S4a and S4b). The results clearly showed that both ASF/SF2 and SRp20 are negative regulators of IL-1β production. As mentioned above, the second step necessary for IL-1β secretion is the processing by the Inflammasome, in a Caspase-1 dependant manner. Caspase-1 activation was previously shown to be induced upon *E.coli* challenge, as measured by FACS. Taking advantage of this assay, we determined the impact of SFRS1 and SFRS3 knockdown in Caspase-1 activity, as an indirect measurement of Inflammasome activation. Knocking-down SFRS1 did not show any impact in Caspase-1 activation. In opposition, a remarkable increase in Caspase-1 activation was observed upon SFRS3 knockdown, as compared to control cells. With these results we could conclude that SRp20 acts as a negative regulator of both IL-1β production and processing, whereas ASF/SF2 is a negative regulator of IL-1β production.

The increased Caspase-1 activation observed upon SFRS3 downregulation, suggested that it could be a result of increased expression of the NALP3 Inflammasome components, as observed for IL-1β and IL-8. Consequently, we evaluated the expression of the NALP3 Inflammasome components (NALP3, ASC and Caspase-1). Increased Caspase-1 mRNA expression was observed in cells targeted for SFRS3 knockdown, as compared with SFRS1 knockdown or control cells. The expression of the other two Inflammasome components, ASC and NALP3, was not altered significantly. The results obtained suggest that the increased Caspase-1 activity observed in SFRS3 knocked-down cells might be due to increased Caspase-1 expression, although further experiments are still needed to be performed to confirm this hypothesis.

The finding of SRp20 as a modulator of Caspase-1 expression and activity is

of great relevance. With the exception of the cytokine substrates (IL-1β, IL-18 and IL-33) little was known, until recently, about the spectrum of cellular proteins targeted by this protease. Several studies have attempted to identify the possible substrates of this protease, and the cellular processes regulated. One such study was conducted *by Shao et al.* (*Shao et al.*, 2007) where 41 proteins were identified to be directly cleaved by Caspase-1. Among the proteins identified, several involved in glycolisis were confirmed as Caspase-1 substrates, therefore implicating Caspase-1 in the regulation of the glycolytic pathway. In addition, several other substrates such as chaperones, cytoskeletal proteins, transcription factors and Caspase-7 were more recently identified as Caspase-1 substrates (*Lamkanfi et al.*, 2008). Moreover, *in vivo* experiments have proven that Caspase-7 processing was Caspase-1 dependant in conditions known to induce Caspase-1 activation, such as after stimulation with LPS and ATP, and abolished in the absence of Caspase-1 (*Lamkanfi et al.*, 2008). Together with Caspase-3 and 6, Caspase-7 belongs to the group of executioner Caspases, for a long time identified to be involved in apoptosis (*Lamkanfi et al.*, 2002). Thus, Caspase-1 controlling Caspase-7 activity, can also control apoptosis in certain cellular conditions (reviewed in *Lamkanfi et al.*, 2010). The involvement of Caspase-1 in certain diseases has also been shown, such as Neurodegenerative and Inflammatory diseases (reviewed in *Howley et al.*, 2008). If in some disease states, the main problematic agent is the increased levels of cytokines released, such as IL-1β, and the treatment of the patients with anti-IL-1β or other methods to decrease circulant levels of this cytokine proves to be quite successful (*Burger et al.*, 2006; *Alten et al.*, 2008), others exist where it might not be thriving, as the case of septic shock. Recently, increased resistance to septic shock was observed in mice lacking Caspase-1 expression, and not in IL-1β/IL-18 double knockout mice (*Li et al.*, 1995; *Sarkar et al.*, 2006). In conclusion, the described roles of Caspase-1 in different cellular functions and diseases, unquestionably shows the importance of

the regulation of this cellular protease. Therefore, the implication of the Splicing factor, SRp20, in the regulation of its activity and expression results in a valuable knowledge that can be used to unveil the mechanisms of Caspase-1 regulation and to the discovery of possible targets for therapeutical studies.

The major Inflammasome involved in IL-1β secretion after *E.coli* challenge was shown to be the NALP3 Inflammasome. However, we could not discard the hypothesis that the increased Caspase-1 activation observed upon knocking-down the expression of SFRS3, was due to the activation of different Inflammasomes. The three main Inflammasomes described so far are the NALP3, NALP1 and IPAF Inflammasomes (reviewed in *Martinon et al., 2009; Stutz et al., 2009; Latz, 2010*), therefore we decided to determine the role of each one of these Inflammasomes in IL-1β secretion upon SFRS3 knockdown. Performing a series of double knockdown experiments targeting SFRS3 and at the same time NALP3, NALP1, IPAF or the common Inflammasome adapter ASC, we could conclude that the effect observed by knocking-down SFRS3 is not due to the activation of the IPAF Inflammasome, since no differences on IL-1β secretion were observed upon SFRS3/IPAF double knockdown. In what concerns the other two Inflammasomes, we observed that performing the double knockdown of NALP1 or NALP3 in addition to SFRS3, a partial reversion of the phenotype was observed; however not as significant as the reversion observed when knocking-down the common adaptor ASC. These results suggested that the increased Caspase-1 activation observed upon SFRS3 knockdown might be also due to different Inflammasomes activation (NALP1 and NALP3, but not IPAF), in addition to the increased Caspase-1 expression described before. Further experiments are now required to evaluate this hypothesis.

A number of studies reported that the activation of the IPAF Inflammasome might also occur independently of the ASC adaptor (*Franchi et al.*, 2007;

Mariathasan et al., 2007; *Case et al.*, 2009); therefore we hypothesized that somehow by knocking-down SFRS3 we could be altering the activation or expression profiles of ASC. As mentioned before, no differences at mRNA expression levels of ASC were observed upon knocking-down SFRS3 and posterior *E.coli* challenge. Although, several Splicing variants of ASC have been predicted to exist (http://www.imm.fm.ul.pt/exonmine), therefore the effect could be due to changes in the Splicing pattern of ASC. In addition to the bioinformatics predictions, *Matsushita et al.* reported recently the existence of a splice variant of ASC that can regulate IL-1β (*Matsushita et al.*, 2009a). We have performed RT-PCR experiments in cells targeted for SFRS3 knockdown and challenged with *E.coli* for different time points, and no differences in the Splicing pattern of ASC were observed in comparison with control (data not shown), thus excluding this hypothesis.

As mentioned previously, the SR family of Splicing factors has been shown to play a role in different cellular processes. A recent report by *Xiong et al.* (*Xiong et al.*, 2006) clearly shows that ASF/SF2 is downregulated in inflamed muscle. Moreover, it was shown that this downregulation could be induced in the presence of pro-inflammatory stimuli, such as TNF-α (*Xiong et al.*, 2006; *Balkwill*, 2009). Consequently, we decided to check the expression of SFRS1 and SFRS3 upon *E.coli* challenge. In agreement to *Xiong's* report, SFRS1 expression was downregulated upon *E.coli* challenge. In opposition, SFRS3 expression did not change significantly at the mRNA level, but was decreased at protein level upon *E.coli* challenge. In addition, we evaluated the expression of the two SF candidates upon *M.tb* infection. As reported previously, we have identified NALP3 as the major Inflammasome activated upon *M.tb* infection of THP-1 cells (*Mishra et al.*, 2010). As can be seen in Figure S5, after *M.tb* infection of THP-1 cells, decreased mRNA expression of both SF candidates is observed, in opposition with the

increased IL-1β expression and secretion, further substantiating the observations obtained after *E.coli* challenge. In future studies, similar approaches can be expanded to primary cells obtained from volunteer donors. In addition, differences in expression of these two SFs can also be evaluated in cells obtained from patients with several inflammatory diseases, such as Crohn's Disease, Rheumatoid Arthritis or Gout, in order to evaluate if differences at the expression levels of these two SF can be linked with Disease.

We decided to go further on studying the downregulation mechanism of our two main candidates, ASF/SF2 and SRp20. Preliminary results obtained in collaboration with *Margarida Carvalho* and *Ana Neves Costa*, suggested that this downregulation can be hnRNPK dependent. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) belongs to the hnRNP family of proteins, which directly interact with DNA and RNA through their K homology (KH) domains and regulate gene expression at multiple levels including transcription, RNA Splicing, RNA stability and translation (reviewed in *Bomsztyk et al., 2004*). Preliminary results obtained by *Bruno d'Almeida* (UBCSI, IMM, unpublished data) show that the SFRS1 mRNA is unstable (*unpublished data*). Moreover, *d'Almeida*'s work also shows that SFRS1 downregulation is ERK1/2 dependent, either by using inhibitors or by performing ERK1/2 knockdown (unpublished data). It is already known that activation of ERK1/2 results in cytoplasmic accumulation of hnRNPK (*Habelhah et al.*, 2001). In addition, increased cytoplasmic expression of hnRNPK has been reported to be able to repress translation of several proteins (*Habelhah et al.*, 2001). All these data, prompted us to look to a possible regulation of SFRS1 and SFRS3 by hnRNPK. As shown in Figure S6, IL-1β secretion is decreased upon hnRNPK knockdown. Moreover, SFRS1 expression after *E.coli* challenge seems to be less downregulated in cells knocked-down for hnRNPK. RNA-IP experiments are being performed in order to understand if hnRNPK can bind SFRS1 RNA and therefore control its expression. The role of hnRNPK in regulating SRp20

expression is also under evaluation.

In addition to hnRNPK, Sam68, other RNA binding protein, is also a promising candidate to be playing a role in the regulation of SFRS1 and SFRS3 after *E.coli* challenge. This RNA-binding protein belongs to the signal transduction and activation of RNA metabolism family characterized by having a KH domain, similar to hnRNPK (*Vernet et al.*, 1997; *Lukong et al.*, 2003). It has been implicated in the regulation of cell cycle and apoptosis, and its subcellular localization and RNA affinity were shown to be regulated by post-translational modifications, such as phosphorylation and methylation (*Paronetto et al.*, 2003; *Taylor et al.*, 2004; *Babic et al.*, 2006; *Paronetto et al.*, 2007). Moreover, like hnRNPK, ERK1/2 phosphorylation was shown to be able to modulate Sam68 activity (*Paronetto et al.*, 2007). Unpublished data from *Dr.Giuseppe Biamonti* (IMG, Pavia, Italy) shows that Sam68 can modulate ASF/SF2 expression, and it is dependant on ERK1/2 phosphorylation of Sam68, as shown using Sam68 inhibitors. In addition, Sam68 was demonstrated to counteract ASF/SF2 dependant Splicing of certain genes, such as Bcl-x (*Paronetto et al.*, 2007). All these information thus seem to suggest that Sam68 can also play a role in IL-1β secretion by counteracting ASF/SF2 effects, although several experiments need to be performed in order to prove this hypothesis.

As referred earlier, the decreased expression of the Splicing factor SRp20 is only observed at the protein level, therefore several hypothesis can be tested in an attempt to identify the underlying mechanism. One of such hypothesis is the possible regulation of SRp20 expression by microRNAs (miRNAs). MicroRNAs are small noncoding RNAs, around 22 nucleotides, which regulate protein-coding genes via posttranscriptional repression (reviewed in *Taganov et al.*, 2007b; *Baltimore et al.*, 2008). Since the discovery of the first miRNA, lin-4 (*Lee et al.*, 1993; *Wightman et al.*, 1993), more than 700 miRNAs have been shown to be important regulators in development, differentiation and homeostasis (reviewed in

Stefani et al., 2008; *Williams*, 2008). Increasing amount of evidence is showing that several Innate and Adaptative Immune responses, as well as inflammatory networks in various cell and tissue types are regulated by these small RNAs (reviewed in *Taganov et al.*, 2007b *Baltimore et al.*, 2008; *Sheedy et al.*, 2008; *Bi et al.*, 2009). A recent report by *Taganov et al.,* has shown that LPS stimulation of THP-1 cells significantly induces the expression of miR-146, miR-132 and miR-155, from a panel of 200 miRNA tested (*Taganov*, 2006). Furthermore, promoter analysis of the miR-146 unveiled that it is regulated by the NF-kB transcription factor and may function as a negative regulator of IRAK-1 and TRAF6 expression, therefore regulating an inflammatory response (*Taganov*, 2006). Another report by *Perry et al*., has shown that miR-146 expression was related to IL-1β induced responses (*Perry et al.*, 2008). All these information lead us to investigate the role of miRNA in Inflammation, and more precisely the possible role in the regulation of SRp20 expression upon *E.coli* challenge. The expression of several SFs has also been shown to be regulated by miRNA expression, as the case of the polypyrimidine tract-binding protein (*Boutz et al.*, 2007). Preliminary results from our lab show that knocking-down the expression of DICER, a component of the microRNA pathway (reviewed in *Taganov et al.*, 2007a), results in remarkably decreased IL-1β secretion after 24 hr *E.coli* challenge; as expected, due to the increase number of genes in this pathway that are regulated by miRNA. The expression of SRp20 was not yet assessed in these conditions, but it will be in the near future to try to disclose if SRp20 expression is being regulated by miRNAs induced upon an inflammatory stimulus.

The expression profile of the other putative Negative Regulator SF candidates was also determined in the outcome of an inflammatory response to *E.coli* challenge. The expression profile of TET1 is similar to the profile observed for SFRS1, it is clearly downregulated at mRNA level upon *E.coli* challenge (Figure S7-e). Therefore, this gene is now also under study for its role in IL-1β

secretion upon *E.coli* challenge, by another colleague in the laboratory, *Ana Neves Costa* (UBCSI, IMM*)*. In what concerns the other candidates, GNB2L1 expression is also downregulated in a similar pattern although not as significantly as TET1 or SFRS1 (Figure S7-a). SFPQ expression is induced at mRNA level (Figure S7-d). MFAP1 and PPIE show a similar kinetics of expression upon *E.coli* challenge: they are both downregulated at 4 hours and then recover their steady-state expression levels around 24 hr post-challenge (Figure S7-b and c). These results thus suggest that in the outcome of an inflammatory response, the expression of these Negative Regulators can be modulated in order to achieve maximum IL-1β secretion.

As *Leeman*, *Lynch, Wells* and others have already reported (reviewed in *Lynch*, 2004; *Wells et al.*, 2006; *Leeman et al.*, 2008) the extent of genes found to play a role in the immune responses that are regulated by AS has been increasing over the past years. Therefore, in an attempt to identify possible ASE that occur upon an *E.coli* challenge and SFRS1 or SFRS3 knockdown, PCR analysis was performed for the genes already described to play a role in IL-1β secretion. The ASE predictions were based on Splicing patterns described for each gene in the Exonmine online Database. Since the results obtained were not conclusive, we performed a high throughput analysis of the Splicing events using the Affymetrix® GeneChip® Human Exon 1.0 ST Arrays platform. This platform has been extensively used to detect Splicing events that occur in different biological processes and diseases such as Cancer (*Gardina et al.*, 2006; *French et al.*, 2007; *Cheung et al.*, 2008); identification of tissue specific isoforms of certain genes (*Clark et al.*, 2007; *Das et al.*, 2007); identification of certain SF dependant ASE (*Hung et al.*, 2008; *Oberdoerffer et al.*, 2008). We performed a number of analysis addressing different questions, namely the identification of ASE that occurs upon *E.coli* challenge and moreover the ASE that are dependent on the ASF/SF2

expression. After data analysis and validation we have identified numerous genes that undergo AS after an *E.coli* challenge (listed on Tables S12 and S15), ASF/SF2 knockdown (Tables S13 and S16) or upon ASF/SF2 knockdown and posterior *E.coli* challenge (Tables S14 and S17). Several genes that were previous reported to undergo AS that play a role in the NF-kB or TLR pathways (reviewed in *Leeman et al.*, 2008) were found in our analysis, therefore validating our approach. Some of the genes in these pathways were found to have different Splicing patterns upon ASF/SF2 downregulation by RNAi and therefore constitute good candidates to further test their ASF/SF2 Splicing dependency and role in IL-1β secretion. In addition to the known candidates, several other candidates not previously described in the literature can be subject to further studies to identify their possible involvement in the regulation of IL-1β secretion in a Splicing dependent manner.

Focusing on the genes whose Splicing as found to be ASF/SF2 dependant, by crossing the data obtained in the array with the data generated by the ASF/SF2 CLIP-Seq analysis performed by *Sanford et al*. (*Sanford et al.*, 2009), we have found common ASE. Several bioinformatic approaches, such as ESEFinder (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home), Splicing Rainbow (http://www.ebi.ac.uk/asd-srv/wb.cgi?method=8) or Rescue-Ese (http://genes.mit.edu/burgelab/rescue-ese/) have tried to predict the genes and the binding sites that the majority of the Splicing factors could bind and therefore regulate their Splicing profiles (reviewed in *Long et al.*, 2009). Although these bioinformatic approaches have generated valuable information, the majority of the predictions and physical interactions could not be proven to exist. This problem is overcomed in the CLIP-Seq analysis since it relies on the identification of RNA sequences that are physically bound to a specific protein. Being cautious that different conditions exist in our experiments and the *Sanford's* study, namely a different cell type (HEK293 vs. THP-1), the results are undoubtly encouraging, since they prove that ASF/SF2 binds to the RNA of several candidate genes

obtained by the Exon array analysis, therefore capable of regulating their Splicing pattern, likewise their cellular functions. Nevertheless, this is still the "tip of the Iceberg" and several experiments are required to determine which Splicing events that are dependant on ASF/SF2 do play a role in the increased IL-1β secretion phenotype found upon the knockdown of this SF.

In an attempt to identify the Splicing events related to the other SR protein, SRp20, a similar high throughput analysis can be done in the future. Perhaps, in spite of performing an Exon-array analysis, it would be of greater interest to perform Deep-sequencing analysis. Deep-sequencing analysis is now accepted as the best method to detect ASE (reviewed in *Pan et al.*, 2008; *Sultan et al.*, 2008; *Wang et al.*, 2009)), thus, this could be performed in similar conditions to what was performed to ASF/SF2. Analogous set of validation would be performed and the most promising candidates studied in more detail for their role in the regulation of IL-1β secretion in an SRp20 dependant manner.

In addition to the ASE predicted by the Exon array analysis, the differences at gene expression level (DEG) could also be evaluated. Several genes were found to be differently expressed in the different analysis performed. This platform proved to be a very good experimental system to detect DEG, since the rates of validation were almost 97%. Our results are in agreement with other published studies that have used the Human Exon 1.0 ST Arrays platform to detect DEG in different conditions (*Huang et al.*, 2007; *Chahrour et al.*, 2008; *Chiao et al.*, 2008; *Duan et al.*, 2008; *Ge et al.*, 2008). A preliminary Bioinformatic analysis was performed, using the Ingenuity® Pathway analysis software, and we could observe that several genes involved in the regulation of an inflammatory response are differentially expressed, as well as different inflammatory responses and pathways activated. In agreement to what was reported previously, IL-1β and IL-8 expression is induced after *E.coli* challenge, whereas SFRS1 expression is

downregulated upon this bacterial challenge. Once more, however these results were expected, this is in agreement to our previous results, therefore validating our overall approach. In the future, the most promising candidates that are differentially expressed in the shSF2-S vs. Control-S analysis will be studied for their possible role in the regulation of IL-1β secretion.

Besides ASF/SF2 and SRp20, several other candidates have been reported to be linked with inflammatory processes. Among the positive regulator candidates, High-mobility group box-1 (HMGB1) has been reported to be an endogenous danger signal molecule (DAMP, reviewed in *Erlandsson Harris et al.*, 2004). A recent report by *Mouri et al.* shows that HMGB1 can act as a transactivator of IL-1β promoter, therefore regulating IL-1β production (*Mouri et al.*, 2007). We performed a qRT-PCR assay to quantify the levels of IL-1β mRNA expression upon HMGB1 knockdown. The downregulation of HMGB1 by RNAi lead to decreased IL-1β mRNA expression and protein secretion (Figure S4-c). Moreover, the reduction at the secretion level is only observed at 24 hours post-challenge, what can be justified by the fact that HMGB1 is thought to be a late mediator of Inflammation (reviewed in *Klune et al.*, 2008), therefore exerting its effect in a later time point after *E.coli* challenge, and not initially. Strong associations between HMGB1 and inflammatory diseases have been extensively reported, such as in the development of Rheumatoid Arthritis and Sepsis (*Wittemann et al.*, 1990; *Wang et al.*, 1999; *Klune et al.*, 2008). Consequently, the identification of HMGB1 as a positive regulator of IL-1β secretion using our methodology, is a proof of principle of our experimental design.

Interestingly, among the other candidates identified in our study, single nucleotide polymorphisms (SNPs) in CUGBP2, SFPQ and hnRNPM have been shown to be associated with Rheumatoid Arthritis Disease (*Consortium*, 2007), however the mechanisms that correlate those SNPs and the disease development

are not known. The strong correlation between increased IL-1β levels and the development of this disease suggested the treatment of these patients with anti-IL-1β antibodies, IL-1R antagonists or by other methods to decrease IL-1β levels (*Burger et al.*, 2006; *Alten et al.*, 2008).

The zinc finger containing protein ZC3H13 was found in the RNAi screen to play a positive role in IL-1β secretion. Recently, several papers have described the contribution of an homolog zinc finger containing protein ZC3H12a as being involved in the regulation of the inflammatory response (*Liang et al.*, 2008a; *Liang et al.*, 2008b; *Matsushita et al.*, 2009). ZC3H12a, like ZC3H13, has a CCCH-type zinc finger motif, and belongs to the family of proteins that bind to the AU-rich elements in mRNA, leading to the removal of the poly(A) tail from that mRNA and increasing rates of mRNA turnover (*Carballo et al.*, 1998; *Lai et al.*, 1999; *Carrick et al.*, 2004). It was shown that ZC3H12A could regulate the stability of different inflammatory genes, therefore controlling its expression (*Liang et al.*, 2008a; *Liang et al.*, 2008b; *Matsushita et al.*, 2009). Among the cytokines whose mRNA levels were shown to be regulated by this protein, is IL-1β (*Liang et al.*, 2008b). Therefore we could speculate that a similar mechanism can be regulated by ZC3H13, although it was beyond the scope of our study.

Chapter 5 - Concluding Remarks

"The most beautiful thing we can experience is the mysterious. It is the source of all true art and science"

Albert Einstein

The results obtained in my PhD work allowed us to identify several novel regulators of IL-1β secretion after *E.coli* challenge. We have focused in two of the candidates, the ASF/SF2 and SRp20, the SFs encoded by the genes SFRS1 and SFRS3, respectively. We have demonstrated that SRp20 plays a role in both Production and Processing steps required for IL-1β secretion, while ASF/SF2 is only involved in the regulation of the first step. The underlying mechanisms are not yet uncovered, nevertheless, the implication of several SF in the regulation of IL-1β secretion is of extreme importance and for the first time described. This discovery is of great relevance for the understanding of the regulation of an inflammatory response and possibly for clinical application, as unveiling putative targets to modulate this response. The increased number of diseases linked to Splicing disregulation definitely marks the importance of this process in cell and organism homeostasis (reviewed in *Tazi et al.*, 2008). Several Splicing regulators are now under intensive studies as targets for the control of several diseases, such as shown by the work of *Dr. Jamal Tazi's* group (*Keriel et al.*, 2009), where specific inhibitors of ASF/SF2 can have a dramatically impact in retroviral pathogenesis. Possibly, such type of studies can be extended to the regulation of these two SFs in the context of inflammatory diseases.

The tight regulation of Splicing regulatory factors, either at concentration, localization, or activity has been revealed important in the maintenance of the organism homeostasis. Several reports have clearly demonstrated specific alterations in the expression patterns of some Splicing factors related with several diseases, such as cancer (reviewed in *Tazi et al.*, 2008; *Cooper et al.*, 2009). In agreement to what was previously reported *(Xiong*, 2006), ASF/SF2 expression is downregulated upon an inflammatory challenge, and we have also found that it occurs for the other SF studied - SRp20. The exact mechanisms by which these SF are being regulated are now under evaluation and will provide a deeply valuable knowledge in the intricate regulatory networks that are involved in the regulation

of an inflammatory response, namely the IL-1β secretion.

The discovery of the involvement of SRp20 in the regulation of Caspase-1 expression and activity is also of great novelty and relevance. In the past years, the number of cellular targets, processes and diseases related to this protease has been increasing. Therefore, unveiling SRp20 as a Caspase-1 regulator can undoubtly lead to new mechanisms of regulation and possibly new therapeutical targets when Caspase-1 activity needs to be tightly regulated, for example during the course of a particular disease.

In conclusion, though the exact mechanisms by which these two SF may play a role in IL-1β secretion are not yet known, our data strongly suggest that they are indeed playing a role, as shown by the various methods applied getting a consistent result, either overexpressing experiments showing an opposite phenotype to what observed by knocking-down or inhibiting the Splicing dependent on these two SFs. Several studies are now required to determine the exact mechanisms by which these two SFs are playing a role in IL-1β secretion.

Chapter 6 - Bibliography

"In science the credit goes to the man who convinces the world, not the man to whom the idea first occurs."

Sir Francis Darwin

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Chapter 7 - Supplementary data

7.1 - shRNA Library subset- NLR and CARD-domain containing proteins.

Table S1 – List of NLR/CARD-domain proteins targeted to knockdown by shRNA.

Gene Name	NM_Id	Gene Name	NM_Id
ASC	NM_013258	**NALP2**	NM_017852
BIRC1	NM_004536	**NALP3**	NM_004895
CARD9	NM_052813	**NALP4**	NM_134444
CARDINAL	NM_014959	**NALP5**	NM_153447
CARMA1	NM_032415	**NALP7**	NM_139176
CARMA2	NM_024110	**NALP8**	NM_176811
CIITA	NM_000246	**NALP9**	NM_176820
CINCIN1	NM_032587	**NOD1**	NM_006092
IPAF	NM_021209	**NOD2**	NM_022162
MDA5	NM_022168	**NOD27**	NM_032206
NALP1	NM_014922	**NOD9**	NM_024618
NALP11	NM_145007	**RIG-I**	NM_014314
NALP12	NM_144687	**RIPK2**	NM_003821
NALP14	NM_176822	**VISA**	NM_020746

7.2 - shRNA Library subset- Splicing Factors and Splicing related proteins (SFs)

Table S2– List of SFs targeted to knockdown by shRNA.

Gene Name	NM_Id	Gene Name	NM_Id
A2BP1	NM_145891	**NOVA1**	NM_006491
ABT1	NM_013375	**NOVA2**	NM_002516
ACIN1	NM_014977	**NUDT21**	NM_007006
ADAR	NM_001111	**NUMA1**	NM_006185
AKAP8	NM_005858	**NXF1**	NM_006362
AQR	NM_014691	**PABPC1**	NM_002568
ARS2	NM_015908	**PABPC4**	NM_003819
ASCC3L1	NM_014014	**PABPN1**	NM_004643
BAT1	NM_004640	**PARP1**	NM_001618
BCAS2	NM_005872	**PCBP1**	NM_006196
BRUNOL4	NM_020180	**PCBP2**	NM_005016
BRUNOL5	NM_021938	**PCBP3**	NM_020528
BRUNOL6	NM_052840	**PCBP4**	NM_020418
BUB3	NM_004725	**PHF5A**	NM_032758

C10orf116	NM_006829	**PIAS1**	NM_016166
C13orf10	NM_022118	**PLRG1**	NM_002669
C14orf166	NM_016039	**PNN**	NM_002687
C1orf55	NM_152608	**POLDIP3**	NM_032311
C1orf60	NM_023015	**POLDIP3**	NM_032311
C1QBP	NM_001212	**POLR2A**	NM_000937
C20orf14	NM_012469	**POLR2B**	NM_000938
C20orf23	NM_024704	**PPIE**	NM_006112
C21orf66	NM_016631	**PPIH**	NM_006347
C21orf70	NM_058190	**PPIL1**	NM_016059
C22orf19	NM_003678	**PPIL2**	NM_014337
C2orf3	NM_003203	**PPIL3**	NM_130906
C9orf10	NM_014612	**PPM1G**	NM_002707
CCDC12	NM_144716	**PPWD1**	NM_015342
CCNA1	NM_003914	**PRCC**	NM_005973
CCNK	NM_003858	**PRKRA**	NM_003690
CD2BP2	NM_006110	**PRPF18**	NM_003675
CDC2L2	NM_024011	**PRPF19**	NM_014502
CDC40	NM_015891	**PRPF3**	NM_004698
CDC5L	NM_001253	**PRPF31**	NM_015629
CHERP	NM_006387	**PRPF38A**	NM_032284
CIRBP	NM_001280	**PRPF38B**	NM_018061
CLK1	NM_004071	**PRPF4**	NM_004697
CLK2	NM_003993	**PRPF4B**	NM_003913
CLK3	NM_003992	**PRPF8**	NM_006445
CLK4	NM_020666	**PSEN1**	NM_000021
CPSF1	NM_013291	**PSIP1**	NM_021144
CPSF2	NM_017437	**PTBP1**	NM_002819
CPSF3	NM_016207	**PTBP2**	NM_021190
CPSF4	NM_006693	**QKI**	NM_006775
CPSF6	NM_007007	**RALY**	NM_016732
CRK7	NM_016507	**RAVER1**	NM_133452
CRNKL1	NM_016652	**RBBP7**	NM_002893
CSDA	NM_003651	**RBM10**	NM_005676
CSN3	NM_005212	**RBM12**	NM_006047
CSTF3	NM_001326	**RBM15**	NM_022768
CTNNBL1	NM_030877	**RBM17**	NM_032905
CUGBP1	NM_006560	**RBM22**	NM_018047
CUGBP2	NM_006561	**RBM25**	NM_021239
CWF19L1	NM_018294	**RBM3**	NM_006743
DDB1	NM_001923	**RBM5**	NM_005778
DDX1	NM_004939	**RBM7**	NM_016090
DDX17	NM_006386	**RBM8A**	NM_005105
DDX19B	NM_007242	**RBM9**	NM_014309
DDX21	NM_004728	**RBMS1**	NM_016836
DDX23	NM_004818	**RBMX**	NM_002139
DDX26	NM_012141	**RBMX2**	NM_016024
DDX39	NM_005804	**RBP7**	NM_052960
DDX3X	NM_001356	**RDBP**	NM_002904
DDX41	NM_016222	**REXO1**	NM_020695
DDX46	NM_014829	**REXO2**	NM_015523
DDX48	NM_014740	**RKHD3**	NM_032246

DDX49	NM_019070	**RNGTT**	NM_003800
DDX5	NM_004396	**RNPC2**	NM_004902
DDX54	NM_024072	**RNPS1**	NM_006711
DDX6	NM_004397	**ROD1**	NM_005156
DEK	NM_003472	**RP13-297E16.1**	NM_005088
DGCR14	NM_022719	**RPL22**	NM_000983
DHX15	NM_001358	**RPL23A**	NM_000984
DHX16	NM_003587	**RPL31**	NM_000993
DHX35	NM_021931	**RPL5**	NM_000969
DHX38	NM_014003	**RPS10**	NM_001014
DHX8	NM_004941	**RPS11**	NM_001015
DHX9	NM_001357	**RPS12**	NM_001016
DIDO1	NM_022105	**RPS13**	NM_001017
DKFZP434K1421	NM_032141	**RPS15**	NM_001018
DNAJC17	NM_018163	**RPS15A**	NM_001019
DNAJC6	NM_014787	**RPS16**	NM_001020
DNAJC8	NM_014280	**RPS17**	NM_001021
EDG2	NM_057159	**RPS18**	NM_022551
EEF1A1	NM_001402	**RPS19**	NM_001022
EFTUD2	NM_004247	**RPS25**	NM_001028
EIF2S2	NM_003908	**RPS29**	NM_001032
EIF3S10	NM_003750	**RPS3**	NM_001005
EIF3S2	NM_003757	**RPS3A**	NM_001006
EIF3S6	NM_001568	**RPS4X**	NM_001007
EIF3S6IP	NM_016091	**RPS4Y1**	NM_001008
EIF4A2	NM_001967	**RPS5**	NM_001009
ELAVL1	NM_001419	**RPS7**	NM_001011
ELAVL2	NM_004432	**RPS8**	NM_001012
ELAVL3	NM_001420	**RPS9**	NM_001013
ELAVL4	NM_021952	**RUVBL1**	NM_003707
EP400	NM_015409	**RUVBL2**	NM_006666
ERCC3	NM_000122	**RY1**	NM_006857
ERVWE1	NM_014590	**S100A8**	NM_002964
ET	NM_024311	**S100A9**	NM_002965
EWSR1	NM_005243	**SAFB**	NM_002967
EXOSC1	NM_016046	**SAFB2**	NM_014649
EXOSC10	NM_002685	**SART1**	NM_005146
EXOSC2	NM_014285	**SDCCAG10**	NM_005869
EXOSC3	NM_001002269	**SF1**	NM_004630
EXOSC4	NM_019037	**SF3A1**	NM_005877
EXOSC5	NM_020158	**SF3A2**	NM_007165
EXOSC7	NM_015004	**SF3A3**	NM_006802
EXOSC8	NM_181503	**SF3B1**	NM_012433
EXOSC9	NM_005033	**SF3B14**	NM_016047
FAM32A	NM_014077	**SF3B3**	NM_012426
FAU	NM_001997	**SF3B4**	NM_005850
FIP1L1	NM_030917	**SF3B5**	NM_031287
FKBP3	NM_002013	**SF4**	NM_021164
FLJ20273	NM_019027	**SFPQ**	NM_005066
FLJ21827	NM_020153	**SFRS1**	NM_006924
FMR1	NM_002024	**SFRS10**	NM_004593
FRG1	NM_004477	**SFRS11**	NM_004768

FUBP1	NM_003902	**SFRS12**	NM_139168
FUS	NM_004960	**SFRS16**	NM_007056
FUSIP1	NM_006625	**SFRS2**	NM_003016
G10	NM_003910	**SFRS3**	NM_003017
GNB2L1	NM_006098	**SFRS4**	NM_005626
GPATC1	NM_018025	**SFRS5**	NM_006925
GRSF1	NM_002092	**SFRS6**	NM_006275
GTF2I	NM_001518	**SFRS7**	NM_006276
GTL3	NM_013242	**SFRS9**	NM_003769
HCFC1	NM_005334	**SIAHBP1**	NM_014281
HDAC2	NM_001527	**SKIV2L**	NM_006929
HIST1H2AC	NM_003512	**SKIV2L2**	NM_015360
HIST1H2BC	NM_003526	**SLU7**	NM_006425
HIST2H2AA	NM_003516	**SMARCA5**	NM_003601
HMGB1	NM_002128	**SMC1L1**	NM_006306
HMGB3	NM_005342	**SMC2L1**	NM_006444
HNRNPG-T	NM_014469	**SMNDC1**	NM_005871
HNRPA0	NM_006805	**SMU1**	NM_018225
HNRPA1	NM_002136	**SNIP1**	NM_024700
HNRPA2B1	NM_002137	**SNRP70**	NM_003089
HNRPA3	NM_194247	**SNRPA**	NM_004596
HNRPAB	NM_004499	**SNRPA1**	NM_003090
HNRPC	NM_031314	**SNRPB**	NM_198216
HNRPCL1	NM_001013631	**SNRPB2**	NM_198220
HNRPD	NM_031370	**SNRPC**	NM_003093
HNRPDL	NM_005463	**SNRPD1**	NM_006938
HNRPF	NM_004966	**SNRPD2**	NM_004597
HNRPH1	NM_005520	**SNRPD3**	NM_004175
HNRPH2	NM_019597	**SNRPE**	NM_003094
HNRPH3	NM_012207	**SNRPF**	NM_003095
HNRPK	NM_002140	**SNRPG**	NM_003096
HNRPL	NM_001533	**SNRPN**	NM_003097
HNRPLL	NM_138394	**SNW1**	NM_012245
HNRPM	NM_005968	**SPEN**	NM_015001
HNRPR	NM_005826	**SPPL3**	NM_139015
HNRPU	NM_031844	**SR140**	XM_031553
HNRPUL1	NM_007040	**SRP19**	NM_003135
HSPA1B	NM_005346	**SRP46**	NM_032102
HSPA5	NM_005347	**SRP68**	NM_014230
HSPA8	NM_006597	**SRP9**	NM_003133
HSPC117	NM_014306	**SRPK1**	NM_003137
HSPC148	NM_016403	**SRRM1**	NM_005839
HTATSF1	NM_014500	**SRRM2**	NM_016333
HYPC	NM_012272	**SSB**	NM_003142
IK	NM_006083	**STK23**	NM_014370
ILF2	NM_004515	**STRBP**	NM_018387
ILF3	NM_004516	**SYF2**	NM_015484
IMP-1	NM_006546	**SYNCRIP**	NM_006372
IMP-3	NM_006547	**TAF15**	NM_003487
IQGAP1	NM_003870	**TAF6**	NM_005641
KHSRP	NM_003685	**TCERG1**	NM_006706
KIAA0773	NM_014690	**TCERG1**	NM_006706

KIAA1008	NM_014953	**TDRD3**	NM_030794
KIAA1160	NM_020701	**TET1**	NM_030625
KIAA1429	NM_015496	**TFIP11**	NM_001008697
KIAA1542	NM_020901	**THOC1**	NM_005131
KIAA1604	NM_020943	**THOC3**	NM_032361
KIAA1967	NM_021174	**THOC4**	NM_005782
KIN	NM_012311	**TIA1**	NM_022173
KPNA2	NM_002266	**TIAL1**	NM_003252
LOC138046	NM_173848	**TNPO1**	NM_002270
LSM1	NM_014462	**TNRC4**	NM_007185
LSM10	NM_032881	**TOP1MT**	NM_052963
LSM11	NM_173491	**TOPORS**	NM_005802
LSM2	NM_021177	**TPR**	NM_003292
LSM3	NM_014463	**TPX2**	NM_012112
LSM4	NM_012321	**TRA2A**	NM_013293
LSM5	NM_012322	**TRNT1**	NM_016000
LSM6	NM_007080	**TTF2**	NM_003594
LSM7	NM_016199	**TUBA1**	NM_006000
LSM8	NM_016200	**TUBB**	NM_178014
LUC7L	NM_018032	**TXNL4A**	NM_006701
MAGOH	NM_002370	**U2AF1**	NM_006758
MATR3	NM_018834	**U2AF1L2**	NM_005089
MBD5	NM_018328	**U2AF1L3**	NM_144987
MFAP1	NM_005926	**U2AF2**	NM_007279
MGC13125	NM_032725	**UBL5**	NM_024292
MGC14151	NM_032356	**UNK**	NM_152302
MGC2803	NM_024038	**USP39**	NM_006590
MGC5509	NM_024093	**VIM**	NM_003380
MKI67IP	NM_032390	**WBP11**	NM_016312
MORG1	NM_032332	**WDR33**	NM_001006622
MOV10	NM_020963	**WDR57**	NM_004814
MSI1	NM_002442	**WDR58**	NM_024339
MSI2	NM_138962	**WTAP**	NM_004906
MYEF2	NM_016132	**XAB2**	NM_020196
NCBP1	NM_002486	**XRCC6**	NM_001469
NCBP2	NM_007362	**XRN2**	NM_012255
NCL	NM_005381	**YBX1**	NM_004559
NDUFA1	NM_004541	**ZC3H13**	NM_015070
NHN1	NM_144604	**ZCCHC8**	NM_017612
NHP2L1	NM_001003796	**ZFP36L1**	NM_004926
NIF3L1BP1	NM_025075	**ZFR**	NM_016107
NONO	NM_007363	**ZMAT2**	NM_144723
NOSIP	NM_015953	**ZNF207**	NM_003457

7.3 - Antibodies used to perform Western-blot and their respective experimental conditions

Table S3– List of Antibodies used to perform WB, dilutions and incubation times.

Antibody	Company	Diluition	Incubation	
			Time	Temperature
ASC	Abnova ®	1/1000	O/N	4°C
ASF/SF2	Abcam ®	1/200	O/N	4°C
β-ACTIN	Abcam ®	1/24000	1 hr	RT
CASP1	Abcam ®	1/1000	O/N	4°C
HMGB1	Abcam ®	1/1000	1 hr	RT
IL-1β	R&D ®	1/1000	O/N	4°C
NALP3	Abcam ®	1/1000	O/N	4°C
SRp20	Abnova ®	1/1000	O/N	4°C
V5	Abcam ®	1/1000	1 hr	RT
α-Mouse	Cell Signaling ®	1/5000	1 hr	RT
α-Rabbit	Cell Signaling ®	1/5000	1 hr	RT

O/N-Overnight; RT-Room temperature

7.4 - Primers used in the PCR experiments

Table S4 – List of primers used in the RT-PCR experiments.

Gene Name	Primer	Sequence
ALAS1	ALAS1_E1_FW	GCGTTTCGTTTGGACTTCTC
	ALAS1_E3-RW	CATCATCTTGGGGCAGTTTT
APAF1	APAF1_FW	AAGGATAATGGTGGCAGCAA
	APAF1_RW	TCACCGTTTGAGACATTCCA
ASC	PYCARD.E1F	GCCGAGGAGCTCAAGAAGTT
	PYCARD.E3R	CTGGTACTGCTCATCCGTCA
ATP2B4	ATP2B4_E20FW	GCAATACCTACCCGATCCCT
	ATP2B4_E21RW	GCTGTGGATGGACTTTTGGT
BCL-X	BCLXS.FOR	TTTGAACAGGATACTTTTGTGGAA
	BCLXALL.REV	GTCATGCCCGTCAGGAAC

BCL-X	BCLXL.FOR	AGCCTTGGATCCAGGAGAAC
	BCLXALL.REV	GTCATGCCCGTCAGGAAC
BCL-X	BCLXBOTH.FOR	TTTGTGGAACTCTATGGGAACA
	BCLXALL.REV	GTCATGCCCGTCAGGAAC
BCL-X	BCL_X-FW	CATGGCAGCAGTAAAGCAAG
	BCL_X-RW	TGCTGCATTGTTCCCATAGA
BCL10	BCL10.E2F	TCCTCTCCTTCTTCCCCATT
	BCL10.E3AR	TGGACTTAACCAGCCTCCAG
BCL10	BCL10.E2F	TCCTCTCCTTCTTCCCCATT
	BCL10.E4R	CAGTGGATGCCCTCAGTTTT
BCL2	BCL2-FW	GTTGCTTTTCCTCTGGGAAG
	BCL2-RW	AGCCTGCAGCTTTGTTTCAT
BCL2A1	BCL2A1.E1F	GAGAATGGATAAGGCAAAACG
	BCL2A1.E2R	TGGAGTGTCCTTTCTGGTCA
BID	BID.E3F	GTGTTTGGCTTCCTCCAAAG
	BID.E4R	TCCGGATGATGTCTTCTTGAC
BIN1	BIN1_H-FW	CCTCCAGATGGCTCCCCTGC
	BIN1_H-RW	CCCGGGGGCAGGTCCAAGCG
CARD9	CARD9.E1F	GCAGAACCCATCAGGAAGTG
	CARD9.E3R	ACAGCTGCGGGTAGTAGAGC
CARD9	CARD9.E4F	GCACCAGAGTGAGGAGAAGG
	CARD9.E6R	GTACCTGGATGTAGGGGCTG
CARD9	CARD9.E6F	CCAACACCATCTTCTCCCTG
	CARD9.E8R	CCTCACACTGGAACACCTGC
CARD9	CARD9.E8F	AGCTGCAGGTGTTCCAGTGT
	CARD9.E13AR	GCAAAATGAGTGCCGCTTA
CASP1	CASP1.E2F	TTATCCGTTCCATGGGTGAA
	CASP1.E5R	GCGGCTTGACTTGTCCATTA
CASP1	CASP1.E6F	TTTGAAGGACAAACCGAAGG
	CASP1.E9R	CATCTGGCTGCTCAAATGAA
CASP1	CASP1.E2F	TTATCCGTTCCATGGGTGAA
	CASP1.E9R	CATCTGGCTGCTCAAATGAA
CASP2	CASP2L-FW	AAACGAGGTTCCTGGTACATCG
	CASP2L-RW	TCCTTGATAAGTGCGTTCACC
CASP2	CASP2S-FW	TGCCCAAGCCTACAGAACAAA
	CASP2S-RW	GCAGCAGTGAACAGAAGGAGG
CASP8	CASP8.E2F	GCCCTTGAGTTGGTCACTTG
	CASP8.E6R	GGGCACAGACTCTTTTCAGG
CASP8	CASP8.E1BF	CACGTGGAGTTAGGCAGGTT
	CASP8.E6R	GGGCACAGACTCTTTTCAGG
CASP8	CASP8.E1CF	ATAGGCCTGTGACGAAGGTG
	CASP8.E6R	GGGCACAGACTCTTTTCAGG
CASP8	CASP8.E6F	TGGAGAAGAGGGTCATCCTG
	CASP8.E9R	GCTCTTCAAAGGTCGTGGTC
CASP8	CASP8.E6F	TGGAGAAGAGGGTCATCCTG
	CASP8.E8AR	GCAAAACCCTCTATATTCGTAGC
CASP9	CASP9.E2F	GCTCTTCCTTTGTTCATCTCC
	CASP9.E7R	CATCTGGCTCGGGGTTACTGC
CD44	CD44.C5F	GCAGCACTTCAGGAGGTTACAT
	CD44.V3R	TCATCAATGCCTGATCCAGA
CD44	CD44.C5F	GCAGCACTTCAGGAGGTTACAT

	CD44.V4R	GTCAAAAGCCCGTGGTGT
CD44	CD44.C5F	GCAGCACTTCAGGAGGTTACAT
	CD44.V5R	TGGTGCCATTTCTGTCTACAT
CD44	CD44.C5F	GCAGCACTTCAGGAGGTTACAT
	CD44.V6R	GTTGCCAAACCACTGTTCCT
CD44	CD44.V7F	ATGCAAGGAAGGACAACACC
	CD44.V9F	CATCACATGAAGGCTTGGAA
CD44	CD44.V7F	ATGCAAGGAAGGACAACACC
	CD44.C7R	CAAGAGGGATGCCAAGATGAT
CD45	CD45.E2F	TCTTAGGGACACGGCTGACT
	CD45.E7R	GGGCTCAGAGTGGTTGTTTC
COP1	COP1.E2F	AAGACCCGAGCTTTGATTGA
	COP1.E3R	ATAGCTGGGTTGTCCTGCAC
COP1	COP1.E2F	AAGACCCGAGCTTTGATTGA
	COP1.E4RDW	TTCTGAACATGGCACCTCTG
EIF4H	EIF4H_E4A'_FW	ACTTCGTGTGGACATTGCAG
	EIF4H_E5_RW	CCTGCCCCCTAAGAAGTCAT
ICEBERG	ICEBERG.FOR	GGCTCGAGTCTTGATTGACC
	ICEBERG.REV	TGAGGCAAGTTGAGGGTCTT
IL-32	IL32_E2_FW	TTGGCTCCTTGAACTTTTGG
	IL32_E5_RW	CTCTGCCAGGCTCGACAT
IL1RN	IL1RN.E1F	TGCTACTTTATGGGCAGCAG
	IL1RN.E6R	TCTCGCTCAGGTCAGTGATG
IL1RN	IL1RNV1.E1F	CTCCTCCTCTTCCTGTTCCA
	IL1RN.E6R	TCTCGCTCAGGTCAGTGATG
INCA	INCA.FOR	GGACTCTCAGCAGGTCCAAC
	INCA.REV	CATCTGGCTGCTCAAATGAA
INCA	INCA.EJ2_3F	CTCAGCAGGTCCAACATCTG
	INCA.EJ3_4R	AATCAAAGTTGATCCTAGGAAGG
IRAK2	IRAK2.E11F	GCCAGAAGTACCTGGAGAAGG
	IRAK2.E13R	TTGATCTCAATTTGCCACGA
IRF7	IRF7_E6FW	CATTCCTGGCACACACACAT
	IRF7_E8RW	AGGGTGACAGGTACGGCTCT
MALT1	MALT1_E6FW	GGTGCCTTATGTGGATTTGG
	MALT1_E9RW	AGGCTGGTCAGTTGTTTGCT
MAP3K5	MAP3K5_E14FW	TGGTGAACACCATTACCGAA
	MAP3K5_E19RW	GTCTGCTGCTTTTCCGTAGC
MDM2	MDM2_2FW	CAGCTTCGGAACAAGAGACC
	MDM2_8RW	AGACAGGTCAACTAGGGGAAA
MEFV	MEFV.E1F	AGAGTACGCCGTGCAGCTC
	MEFV.E3R	TCAACTGGGTCTCCTTCCTG
MEFV	MEFV.E3F	CATCTGCCTCATCTGCAGTC
	MEFV.E7R	GGGACAGGCACTGTCTTAGC
MEFV	MEFV.E7F	GTGCCTGTCCCTGAAAAGTG
	MEFV.E10R	CCTCTCCCACTTGTTTCCAA
MKNK2	MKNK2_11_F	CCAAGTCCTGCAGCACCCCTG
	MKNK2_13A_R	GATGGGAGGGTCAGGCGTGGTC
MKNK2	MKNK2_11_F	CCAAGTCCTGCAGCACCCCTG
	MKNK2_13B_R	GAGGAGGAAGTGACTGTCCCAC

MUC2	MUC2.E1F	GCTCGGAGCTCCAGACAg
	MUC2.E3R	TGTAGGCATCGCTCTTCTCA
MyD88	MYD88.E1F	GACTGCTCGAGCTGCTTACC
	MYD88.E4R	ACATTCCTTGCTCTGCAGGT
NALP1	NALP1_FW	TCCTGCCTGCAAACTCATAC
	NALP1_RW	GGATTCGCACAAACTGGAAT
NALP1b	FW-NALP1B	ATTCCAGTTTGTGCGAATCCA
	RW-NALP1B	GTTCCTTGGGGAGTATTTCCAG
NALP3	NALP3.E1F	CGAGGGGTCAGACAGAGAAG
	NALP3.E3R	TTCAATGCACTGGAATCTGC
NALP3	NALP3.E3F	TAACATGCCCAAGGAGGAAG
	NALP3.E7R	ATCCCCTTGTCTCCGAGAGT
NALP3	NALP3.E5F	AGCACCTGTTGTGCAATCTG
	NALP3.E9R	CCGTTTCCACTCCTACCAAG
NFKB2	NFKB2.E11F	TTCTGCAACTGAAACGCAAG
	NFKB2.E16R	CAGCAGAAAGCTCACCACAC
NFKB2	NFKB2.E18F	ACCTTTGCGGGAAACACAC
	NFKB2.E23R	CCGTACGCACTGTCTTCCTT
NFKBIA	NFKBIA.E1F	GCCTGGACTCCATGAAAGAC
	NFKBIA.E3R	GTCTCGGAGCTCAGGATCAC
NFKBIA	NFKBIA.E3F	CTACACCTTGCCTGTGAGCA
	NFKBIA.E5R	GGCAGCATCTGAAGGTTTTC
NFKBIB	NFKBIB_E1FW	CAAAGCCCAGCTACAGGC
	NFKBIB_E2RW	AGGTCATTCTGCAGGTCCAT
NFKBIB	NFKBIB_E1AFW	CATCAGCATGAACCCTTCCT
	NFKBIB_E2RW	AGGTCATTCTGCAGGTCCAT
NFKBID	NFKBID.E9F	CTGCAAATGGGTGCTAATCA
	NFKBID.E10R	AGCAGCAGCTGAACCAGAGT
NLRX1	NLRX1.E9F	CCTTCCCTGGAACTGCTACA
	NLRX.E10R	GGATGAGGAGAAAGCACCAA
OS9	OS9_E12_FW	AGAGGGTTGTCCCCAAAAAG
	OS9_E14_RW	TCGTGTCCTCATCAGTCAGC
P2X4	P2X4-FW	GCTACCAGGAAACTGACTCCG
	P2X4-RW	GGTATCACATAATCCGCCACAT
P2X7	P2X7.E1F	CTCGGATCCAGAGCATGAAT
	P2X7.E3R	TGCTCTTGGCCTTCTGTTTT
P2X7	P2X7.E6F	GCCGAAAACTTCACTGTGCT
	P2X7.E11R	TGTCGATGAGGAAGTCGATG
PANX1	HPANX1_FW	CCACGGAGCCCAAGTTCAA
	HPANX1_RW	CCGCCCAGCAATATGAATCC
PANX2	HPANX2_FW	AAGCGCGAGATCATCGAGAAC
	HPANX2-RW	GCACAGGTAGGAGATGGGC
PANX3	HPANX3_FW	TCATCAGCGAACTGGACAAATC
	HPANX3_RW	GTATCGTTCTTTCCGAGCCTTC
POLR1D	POLR1D_E1_FW	CGATGGAAGAGGATCAGGAG
	POLR1D_E2_RW	ACGCCTTTCTGAAGGACAGA
PTEN	PTEN.E1F	GCAGCTTCTGCCATCTCTCT
	PTEN.E4R	GCAATTAAATTTGGCGGTGT
PTEN	PTEN.E2F	GCAGAAAGACTTGAAGGCGTA
	PTEN.E5R	GCTGTGGTGGGTTATGGTCT
PTEN	PTEN.E5F	TAAAGGCACAAGAGGCCCTA
	PTEN.E7R	GGGTCCTGAATTGGAGGAAT
PTEN	PTEN.E7F	CCGTTACCTGTGTGTGGTGA

	PTEN.E9R	GCTAGCCTCTGGATTTGACG
PVRL2	PVRL2_E5_FW	TCGTCTGCACAGTCACCAAT
	PVRL2_EW1_RW	AGCCACAGCAGTAGCAATGA
RASSF5	RASSF5_E1FW	CCGTACCCGCTACTATTGGA
	RASSF5_E3RW	TCTCGCGTGTTGTAGCTGTC
RASSF5	RASSF5_E2AFW	GAACTCCGGGGTAGATGACC
	RASSF5_E3RW	TCTCGCGTGTTGTAGCTGTC
RET	RET.E3F	AGCTCTGCTTCCCAGAGACA
	RET.E5R	TGATGCAGGTACCACGTCTG
RGS18	RGS18_E2_FW	GTTTCATGAAGACACCCGCT
	RGS18_E5_RW	GCATCAAAACTGTGGAGGGT
RGS8	RGS8_E2FW	AACACCTTAACCCGAAGCCT
	RGS8_E3RW	GGTTGGGTTTGTCTGGAAGA
RIPK2	RIPK2_E1FW	AGTCAGCTCTGGTTCGGAGA
	RIPK2_E1ARW	CGGGGATGACGTATTTTCAC
RIPK2	RIPK2_E1FW	AGTCAGCTCTGGTTCGGAGA
	RIPK2_E3RW	AGGATGCGAAATCTCAATGG
RIPK2	RIPK2_E7FW	GAACTTGAACCAGTTTTGAGAACA
	RIPK2_E7ARW	AAGAGAAGTGCGAGAACCCA
RIPK2	RIPK2_E7FW	GAACTTGAACCAGTTTTGAGAACA
	RIPK2_E8RW	GGTGAATGGCACTTGAAACA
RPS6KB1	RPS6KB1_1_F	AGACAGGGAAGCTGAGGACA
	RPSKB1_R	TGTCTGAGGATTTGCTGTGC
RPS6KB1	RPS6KB1_2_F	GGACGCTGGAGAAGTTCAAG
	RPS6KB1_R	TGTCTGAGGATTTGCTGTGC
SERPINB2	SERPB2.FOR	CAGATGAAATTGCCGATGTG
	SERPB2.REV	TTGCTGGTCCACTTGTTGAG
SFRS1	ASF.E2_3F	TCTCTGGACTGCCTCCAAGT
	ASF.E4R	GGCTTCTGCTACGACTACGG
SHD	SHD_E4FW	CAGTCCAGAGTGGGAGAGGA
	SHD_E6RW	ATTTTCTAGGCCATGGGAGG
SPPL3	SPPL3_E2_FW	TGGTGGATTCCAGTCAAGTG
	SPPL3_E4_RW	CAATTGGAAGGAACAGAGCC
STAT5A	STAT5A_E3FW	TACTTGGCCCAGTGGATTGA
	STAT5A_E4RW	TAGTGCCCCAGCTTGATCTT
STK40	STK40_E1FW	GCTCTGGGCTACGGACTATG
	STK40_E3RW	GGATGAATGGTCCAGCTCTC
TBXAS1	TBXSA1.E9F	AACCCTGACTGCCAAGAGAA
	TBXSA1.E11R	TTTCAGGGTTGAAGGTCTCC
TNFAIP2	TNFAIP2.E1F	GTGTTCTGCGTCTTCACCAA
	TNFAIP2.E4R	GGTCCATCAACTCCCTCACA
TXNDC11	TXNDC11_E5_FW	CCAGCCTCCTGGTTATTTGA
	TXNDC11_E6_RW	GGATACCAGTTTCGCAAGATG
VILL	VILL_E1FW	AGGTAGCCAGGTGTCGGTCT
	VILL_E3RW	GTGGCCTTCGGGCTCT
ZNF692	ZNF692_E1_FW	GAGAGGCACCGTTTCTTCTTA
	ZNF692_E2_RW	CTCCTTGAGGAGGCACCACT

7.5 - Primers used in the qRT-PCR experiments

Table S5 – List of primers used in the qRT-PCR experiments.

Gene Name	Primer	Sequence
AKAP8	AKAP8_FW	CATGCACATGGCCTCTTACG
	AKAP8_RW	GCCTCCTTCCTTGGACATCAT
ASC	ASC_RT_FW	CGAGGAGCTCAAGAAGTTCAA
	ASC_RT_RW	TCTCCAGGTAGAAGCTGACCA
CASP1	CASP1_FW	TCCAATAATGGACAAGTCAAGCC
	CASP1_RW	GCTGTACCCCAGATTTTGTAGCA
CCL2	CCL2_FW	CCCCAGTCACCTGCTGTTAT
	CCL2_RW	AGATCTCCTTGGCCACAATG
CCL20	CCL20_FW	TTTATTGTGGGCTTCACACG
	CCL20_RW	GATTTGCGCACACAGACAAC
CCL4	CCL4_FW	CTGTGCTGATCCCAGTGAATC
	CCL4_RW	TCAGTTCAGTTCCAGGTCATACA
CCL4L2	CCL4L2_FW	CTTCCTCGCAACTTTGTGGT
	CCL4L2_RW	GACTTGCTTGCCTCTTTTGG
CLK1	CLK1.FOR	GAACAAGCGCTGCAAATACA
	CLK1.REV	TGTCCAGGTTCACATCCTTG
CLK4	CLK4.FOR	GAAGTCACAAGCGGAAGAGG
	CLK4.REV	TCCCGATAATCTCGCTCATT
CUGBP2	CUGBP2.FOR	TGAGAACGACATCAGGGTGA
	CUGBP2.REV	GCCATTGCCCTTGTAGAAAA
CWF19L1	CWF19L1.FOR	CCAAGTGTGTGGGGAACTTT
	CWF19L1.REV	AGGTCTTTTCCAAAGCAGCA
CX3CR1	CX3CR1_FW	CCCTGAATCAGTGACAGAAAACT
	CX3CR1_RW	ACGGAGTAGAATATGGACAGGAA
CXCL10	CXCL10_FW	CCAATTTTGTCCACGTGTTG
	CXCL10_RW	TTCTTGATGGCCTTCGATTC
CXCR4	CXCR4_FW	TACACCGAGGAAATGGGCTCA
	CXCR4_RW	AGATGATGGAGTAGATGGTGGG
DDX54	DDX54.FOR	GCTCAAGTGGGACCGTAAGA
	DDX54.REV	GGTCTCGCTTGTAGGAGCTG
DHX38	DHX38.FOR	CTTCCAGAAGGCTCCAGATG
	DHX38.REV	AACAAACATGATGCCGTCAA
EIF3S10	EIF3S10.FOR	AGGACTCTCGTTGGGGAGAT
	EIF3S10.REV	CGTCTCCACTCCTTCTCTGG
ERLIN1	ERLIN1_FW	CTGTGTACTACAGGGGAGGAG
	ERLIN1_RW	GACCCCACCACTTGTTCCAC
GAPDH	GAPDH_F	GAGTCAACGGATTTGGTCGT
	GAPDH_R	TTGATTTTGGAGGGATCTCG
GNB2L1	FW-GNB2L1	GACCATCATCATGTGGAAACTGA
	RW-GNB2L1	CCGTTGTGAGATCCCAGAGG
GTF2I	GTF2I.FOR	TCAGCTCTCGAGTCCATGTG
	GTF2I.REV	GCACGTCCTCTTTCAGTTCC
HMGB1	HMGB1.FOR	TTGCTTTTGCCCATTTTGA
	HMGB1.REV	GCAGGGTGTGTAGACAAAAGC

HMOX1	HMOX1_FW	CAGTGCCACCAAGTTCAAGC
	HMOX1_RW	GTTGAGCAGGAACGCAGTCTT
HNRNPK	HNRPK.F	ACCTAGAGGGGGAGACCTCA
	HNRPK.R	TTGCAGAGTCCCAAGTTTCA
HNRPM	HNRPM.FOR	AGCTGCGGAAGTCCTAAACA
	HNRPM.REV	CCAGTCGTAGCCATCACCTT
HYPC	FW-HYPC	AGACACAAGTCGAATAGTCCTGA
	RW-HYPC	CTGGGGAACGGTTAGGGAG
ICAM1	ICAM1_F	TCTGTGTCCCCCTCAAAAGTC
	ICAM1_R	GGGGTCTCTATGCCCAACAA
IL1B	IL1B_F	CTCGCCAGTGAAATGATGGCT
	IL1B_R	GTCGGAGATTCGTAGCTGGAT
IL8	IL8-FW	TTTTGCCAAGGAGTGCTAAAGA
	IL8-RW	AACCCTCTGCACCCAGTTTTC
IL8RB	IL8RB_FW	GGCCCTGCCTGTCTTACTTTT
	IL8RB_RW	CATCCGCCAGTTTGCTGTAT
KHSRP	KHSRP.FOR	TGGGGATCCTTACAAAGTGC
	KHSRP.REV	CCGAGATCCGTACTCATTCC
KIN	KIN.FOR	GAAGGGAAGGAACAGGAGGT
	KIN.REV	AAGATGTTGCTCCGGATGAG
LAMP3	LAMP3_FW	ACCACCAGCCCAATTACCTAC
	LAMP3_RW	AAGCTAGGGCCGACTGTAACT
LSM7	LSM7.FOR	GACAAGACGATCCGGGTAAA
	LSM7.REV	CAGGGTCTCGCATGTACTCA
MFAP1	MFAP1-FW	CTATGCCCCTATGGAGTCCTC
	MFAP1-RW	GGTCACTGGATGAATCCTCCTC
NALP3	NALP3_RTIME_RW	TCGGCTCATCTCTTTTTGCT
	NALP3_RTIME_FW	AGCCACGCTAATGATCGACT
NFE2	NFE2_FW	GCAGGAACAGGGTGATACAGC
	NFE2_RW	CTCACTTGGAGCATTCAGACC
NUDT7	NUDT7_FW	GACCAGCATTACGTCACACG
	NUDT7_RW	CGTCATTCCCTTGATCTGGT
P2RX1	P2RX1_FW	GGCACTGCAGACCCATCTAT
	P2RX1_RW	TAGTTGGTCCCGTTCTCCAC
P2RY2	P2RY2_FW	TGGTCTATTACTACGCCCGC
	P2RY2_RW	TGCTGCAGTAAAGGTTGGTG
PABPN1	PABPN1.FOR	TGTGGTTCAGTCAACCGTGT
	PABPN1.REV	TAAGGCCAAGGAAGTCCTCA
PPIE	PPIE_FW	ACCAAGCGCGTCTTGTACG
	PPIE_RW	GCATCCTCTGCCAACTCAAAT
PPIH	PPIH_FW	AAGTGCGATTGGCTGGATGG
	PPIH_RW	ACTGCGAGATCACCACAGGTA
RIPK3	RIPK3_FW	AACCGAACCATCACTCGTGTA
	RIPK3_RW	CCTTCTAAGCCGGGAGTCTCA
SCAMP5	SCAMP5_FW	AAGTGAACAACTTCCCACCATT
	SCAMP5_RW	TGACATGCTGGGGAGGAATATC
SELPLG	SELPLG_FW	CTGCTGCAAGGCGTTCTACT
	SELPLG_RW	GGACAGGTTCCCCATGTTGG
SERPINB2	SERPINB2_FW	CAGCACCGAAGACCAGATGG
	SERPINB2_RW	CCTGCAAAATCGCATCAGGATAA
SF3A3	SF3A3.FOR	GGCACAAAACTTGGTGGAGT
	SF3A3.REV	CCAGCTTCTCAGATGCCTTC
SFPQ	SFPQ-FW	TTCTGGCCTCCTGAACCATAG

	SFPQ-RW	GTTACAGCCGAATGGGCTACA
SFRS1	SFRS1_F	GCGACGGCTATGATTACGAT
	SFRS1_R	CTTGGAGGCAGTCCAGAGAC
SFRS12	SFRS12.FOR	CGCGAAGATCTCGTAGTTCC
	SFRS12.REV	GGGGGACGGAGATCTAGAAG
SFRS2	SFRS2.FOR	CCCGGACTCACACCACAG
	SFRS2.REV	ACCTGGAACGACTCCGACT
SFRS3	SFRS3_F	AACAAGACGGAATTGGAACG
	SFRS3_R	TGGGCCACGATTTCTACTTC
SKIV2L2	SKIV2L2.FOR	TCTGCGCTGTAGCAAAGAGA
	SKIV2L2.REV	AAACTGGGACAACCTGCATC
SNIP1	SNIP1.FOR	TTGATCACCCGTCTTGTTCA
	SNIP1.REV	CCTGAGCCAAGGTCAATGAT
SOD2	SOD2_FW	CTGCTGGGGATTGATGTGTGG
	SOD2_RW	TGCAAGCCATGTATCTTTCAGT
TBC1D30	TBC1D30_FW	TTGACTGGGACAAAACCATGC
	TBC1D30_RW	CTACAGCCTGTGCGGTGAA
TET1	FW-CXXC6	TAATGGAAGCACTGTGGTTTGT
	RW-CXXC6	GCCCCAGATTTGATCTTGGC
TNF	TNF_FW	AACCTCCTCTCTGCCATCAA
	TNF_RW	GGAAGACCCCTCCCAGATAG
TNFAIP6	TNFAIP6_FW	TGGATGGCTAAGGGCAGAGTT
	TNFAIP6_RW	GCGTGTGGGTTGTAGCAATA
VLDLR	VLDLR_FW	AGAACATGCCGCATACATGAA
	VLDLR_RW	GTGAACTCGTCGGGACTACA
ZC3H13	ZC3H13.FOR	TAAAGCGTGGAGAACCCAGT
	ZC3H13.REV	TGAATGGACCTGTCGATCTG

7.6 - DEG genes for the different analysis of the Human Exon 1.0 ST Arrays

Table S6 – List of upregulated genes of Control-S vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ABCC1	F8A3	MOBKL2C	SCN1B
ABTB2	FBXO6	MMP14	SDC4
ACSL1	FCAR	MRAS	SEMA7A
ACVR2A	FEZ1	MSC	SERPINB9
AHDC1	FGF2	MT1A	SERPINE1
AKT1S1	FGL2	MT1B	SERPINE2
ALAS1	FGR	MT1DP	SERTAD1
AMOTL2	FLVCR2	MT1E	SGK1
AMPD3	FMNL3	MT1F	SGPP2
ANXA2	FTH1	MT1H	SH2B3
ANXA8L1	FZD7	MT1M	SHROOM3

APOBEC3F	G0S2	MT1X	SIAH2
APOL1	GADD45B	MT2A	SIRPA
APOL2	GBP1	MTF1	SLAMF1
APOL6	GBP2	MTSS1	SLAMF7
AQP1	GBP3	MUC1	SLAMF8
ARL17	GBP4	MX1	SLC11A2
ARL5B	GBP5	MX2	SLC15A3
ASTL	GCH1	MYD88	SLC16A3
ATF3	GEM	MYO1C	SLC25A25
ATF5	GLT8D4	NAB2	SLC25A28
ATP10A	GMPR	NAV3	SLC2A6
ATP1B1	GNG4	NCOA7	SLC31A2
ATP2B1	GPC4	NFATC1	SLC39A8
ATP9A	GPR132	NFE2L3	SLC41A2
AXUD1	GPR157	NFKB1	SLC43A2
B4GALT1	GPR84	NFKB2	SLC6A6
BAIAP2L1	GRAMD1A	NFKBIA	SLC7A11
BATF	H2AFB3	NFKBIB	SMAD3
BATF2	HCK	NFKBID	SMAD7
BCL2A1	HERC6	NFKBIE	SMOX
BCL2L1	HESX1	NFKBIZ	SNAI1
BCL3	HIVEP1	NINJ1	SNF1LK
BCL6	HIVEP2	NKD1	SNN
BCL9L	HIVEP3	NKX2-2	SNX11
BID	HLA-E	NPTX2	SOCS1
BIRC3	HLA-F	NR4A1	SOCS3
BTG1	HMOX1	NR4A2	SOD2
BTG2	HRH1	NR4A3	SP110
C10orf10	HS3ST3B1	NRP2	SPHK1
C17orf96	ICAM1	NXF1	SPI1
C19orf61	ICOSLG	OAS1	SPIB
C19orf66	IER3	OAS2	SPRED2
C1orf122	IER5	OAS3	SPSB1
C1orf38	IFI16	OASL	SQSTM1
C1orf61	IFI27	OBFC2A	SRC
C3	IFI35	OLIG1	SRXN1
C3AR1	IFI6	OPTN	ST3GAL2
C6orf145	IFIH1	OR6N2	STARD10
CCDC57	IFIT1	OSGIN1	STARD5
CCDC85A	IFIT2	P2RX4	STAT2
CCL1	IFIT3	PARP12	STAT4
CCL18	IFIT5	PAX5	STAT5A
CCL2	IFITM1	PDE4B	STEAP4
CCL20	IFITM3	PDGFRL	STX11
CCL22	IFNGR2	PEA15	TAP1
CCL24	IGFBP3	PHF15	TAPBP
CCL3	IKBKE	PHLDA1	TCEB3
CCL3L1	IL10RA	PHLDB1	TFAP2A
CCL3L3	IL12B	PIK3AP1	TGFBR3
CCL4	IL15RA	PIK3R5	THBS1
CCL4L1	IL18RAP	PIM1	TICAM1
CCL4L2	IL1A	PIM2	TJP2

CCL5	IL1B	PITPNA	TLR7
CCL8	IL1RN	PLA2G4C	TMEM106A
CCR7	IL23A	PLAU	TMEM138
CCRL2	IL27	PLAUR	TMEM140
CD274	IL27RA	PLEK	TMEM171
CD276	IL2RG	PLEKHA4	TMEM2
CD40	IL32	PLEKHO1	TMEM217
CD44	IL33	PLEKHO2	TNF
CD70	IL4I1	PLK3	TNFAIP2
CD80	IL6	PML	TNFAIP3
CD83	IL7R	PNKD	TNFAIP6
CDGAP	IL8	PNPLA1	TNFAIP8
CDKN1A	INDO	PNRC1	TNFRSF10B
CFB	IRAK2	POLR2J	TNFRSF10D
CFLAR	IRF1	POLS	TNFRSF9
CHST2	IRF4	POPDC2	TNFSF10
CKB	IRF7	POU2F2	TNFSF9
CLIP2	IRG1	PPAP2A	TNIP1
CMPK2	ISG15	PPIF	TNIP3
CNP	ISG20	PPP1R15A	TOM1
CNTNAP1	JMJD3	PRDM1	TOR1B
CPM	JUN	PRIC285	TP53INP2
CRIM1	JUNB	PSTPIP2	TP63
CSF1	JUND	PTAFR	TRADD
CTGF	KCNJ2	PTGER2	TRAF1
CXCL1	KCNK5	PTGER4	TRAF3
CXCL10	KCNN4	PTGIR	TRAF3IP2
CXCL11	KIAA0247	PTGS2	TRIB1
CXCL2	KIAA0999	PTPN1	TRIM16L
CXCL3	KLF10	PTX3	TRIM21
CXCL9	KLF5	QPCT	TRIM22
CYB5R2	KYNU	RAB6IP1	TRIM25
CYBASC3	LACTB	RAP2C	TRIM26
CYP19A1	LAMP3	RAPGEF1	TRIM5
CYP27B1	LAP3	RAPH1	TRIM56
CYP7B1	LBH	RARG	TRIP10
DDIT4L	LFNG	RASA4	TSLP
DDX58	LHX2	RASGEF1B	TTC39A
DHX58	LIF	RASGRP1	TYMP
DIXDC1	LIMD2	RASL11A	UBE2L6
DKFZP564O0823	LITAF	RASSF4	ULBP2
DLL4	LPAR1	RASSF5	UPB1
DNAJB5	LPL	RBM24	UPP1
DOT1L	LRG1	RCAN1	USP12
DRAM	LRRC32	RCN1	USP18
DUSP1	LTA	REC8	USP41
DUSP16	LYN	REL	UXS1
DUSP2	LYRM4	RELB	VCAM1
DUSP4	MAFB	RFTN1	VEGFC
DUSP5	MAFF	RGL1	VSNL1
DUSP6	MAGI1	RGS16	WARS
DUSP8	MAML2	RHOG	WTAP

EBI3	MAMLD1	RILPL2	XAGE1A_1
ECE1	MAP2K3	RIN2	XAGE1E
EGR2	MAP3K8	RIPK2	YRDC
EGR3	MARCKS	RND3	ZC3H12A
EHD1	METRNL	RNF144B	ZC3H12C
EHD4	METTL1	RNF19B	ZC3HAV1
ELOVL7	METTL10	RRAD	ZFP36
EMP2	MFSD2	RSAD2	ZMIZ2
EPSTI1	MGLL	RTP4	ZNF8
ETV7	MMP1	SAT1	ZNFX1

Table S7 – List of upregulated genes of shSF2-NS vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ADRBK1	DYNLRB1	MICB	S100A8
ALAD	FAM120C	NCSTN	SLPI
ASB12	GNAI2	OSGEP	TCTA
B3GNT7	GNMT	P2RX1	TMED4
C16orf14	HCCA2	PCBD1	TSPYL1
C3	HSD17B10	PERLD1	U2AF1L4
C3orf60	ITGA5	PPP1R14C	YIPF3
CHCHD10	LILRA2	RHOG	
CHMP6	MANSC1	RIPK3	

Table S8 – List of upregulated genes of shSF2-S vs Control-S analysis.

Gene Name	Gene Name	Gene Name	Gene Name
C3orf60	DUSP2	LRRC32	RNF144B
C6orf58	DYNLRB1	MAGEF1	SEC31B
CCL22	F11R	NCSTN	STAT5A
CCR7	GNAI2	NFKB2	STEAP4
CD83	GPR157	NR4A1	THBS1
CERCAM	HCCA2	NUP210	TMEM158
CTGF	HLA-DOB	OR2J2	TP53INP1
CTSD	HSD17B10	P2RX1	TP53INP2
DDIT4L	INDO	PHLDA3	ZDHHC12
DEFB114	ITGA5	QSOX1	
DUSP1	LAMP3	RAC2	

Table S9 – List of downregulated genes of Control-S vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
AARS	DEPDC6	MID1IP1	RNF125
ACACB	DGCR2	MIF4GD	RNF166
ADCY7	DGKG	MLNR	RNF26
ADCY9	DYSFIP1	MMACHC	RNF44
ADRBK1	EBF3	MOBKL2A	RPUSD3
AKAP1	EBP	MON1A	RWDD2B
ALAD	ECH1	MOSC1	SASH3
ALG3	EHMT2	MPI	SCAMP5
ALKBH2	EPHB1	MRM1	SELPLG
AMPD2	ERLIN1	MRPS34	SERPINB2
ANKRD13D	EXDL2	MTMR4	SFXN3
APEX1	F2R	MUM1	SIGLECP3
ARHGAP1	FAM134A	MXI1	SIPA1
ARHGAP30	FAM163A	MYC	SIRPB2
ARHGEF17	FANCE	MYO18A	SLC12A9
ASB13	FANCF	NAT11	SLC19A1
ATG9A	FARSA	NCAPD2	SLC1A5
B3GNT1	FES	NDRG1	SLC22A5
B3GNT7	FGD3	NDUFS2	SLC25A10
B3GNT8	FLI1	NFE2	SLC25A11
B4GALT3	FLRT1	NLRX1	SLC29A1
BCR	FOXRED2	NMRAL1	SLC2A1
BMP8A	FUT7	NTSR1	SLC2A5
BMP8B	GFI1	NUDCD3	SLC39A3
BRD3	GGA2	NUDT7	SLC43A1
BRI3BP	GIMAP6	OAF	SLC46A1
BSPRY	GIPC1	OBFC2B	SLC6A10P
C10orf140	GIT1	OPRS1	SLC6A8
C10orf54	GLT25D1	ORAI3	SLC9A3R1
C10orf91	GNB5	OXCT2	SORD
C12orf52	GPA33	P2RY2	SSH3
C15orf58	GPR114	P2RY8	ST14
C15orf61	GPT2	PAFAH2	STAR
C16orf53	GRAMD4	PARP15	SYK
C16orf74	GRK6	PCK2	TBC1D14
C16orf88	HAL	PCYOX1L	TBC1D30
C17orf62	HDAC5	PCYT2	TFAP4
C19orf59	HEYL	PDK2	TGM5
C1orf162	HK2	PEX6	THRA
C21orf56	HMHA1	PFKFB4	TLR9
C21orf59	HPS4	PGPEP1	TMEM104
C22orf9	HRH2	PHACTR3	TMEM173
C2orf7	IL8RB	PIK3C2B	TMEM185A
C9orf100	IMPA2	PIPOX	TMEM37
CA2	ING4	PLCB2	TMEM53
CABC1	INPP5D	PLCXD1	TMEM91
CAMK2G	IRX3	PLK1	TMEM97
CARHSP1	ITGAL	PNPO	TNFRSF10A
CBFA2T3	ITPK1	POLG2	TPCN1

CCRK	KANK2	POU4F2	TSC22D3
CD244	KCNAB2	PRAM1	TSHZ1
CD300A	KIAA1462	PRAME	TST
CD300LF	KIF3C	PREB	TTC7A
CD33	KLF16	PRICKLE1	TTLL1
CD93	KLHDC3	PSRC1	TTLL12
CDA	LAIR1	PSTPIP1	UAP1L1
CDC20	LAIR2	PTCH1	UCK2
CDC25B	LDLRAP1	PXMP4	UHRF1
CDIPT	LGALS12	PYCARD	UNC84B
CECR5	LILRA2	RAB11FIP1	VAMP8
CIDEB	LIMK2	RAB33A	VLDLR
CLCN7	LOC116236	RAB3D	VPS26B
CNPY3	LOC201164	RAC2	WDR68
COG8	LOC440295_1	RASGRP2	WDR74
CORO1A	LPAR5	RASSF7	WDR91
CORO2A	LPCAT4	RCSD1	XTP3TPA
CRIPAK	LYL1	REEP4	XYLT2
CSK	LYPLA3	RGS14	YIPF6
CSPG4	MAGEF1	RGS19	YPEL2
CTDSP2	MAP3K3	RHOBTB2	YPEL3
CX3CR1	MCAT	RIMBP3B	ZFYVE1
CXCR4	METTL7B	RIMBP3B	ZMYM3
CXXC1	MFNG	RIMBP3C	ZNF395
DENND2D	MFSD3	RIMS3	ZNF692

Table S10– List of downregulated genes of shSF2-NS vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ACSF2	CCL3L3	GPT2	SCD
ACY1	CD209	LSS	SFRS1
AHNAK2	CREB3L4	NDRG1	SLC43A1
ALDOC	DHCR7	NEU1	SLC6A10P
ATF5	EBP	NKG7	SLC6A8
BHLHB2	ESPL1	OR9Q1	TBC1D30
C1orf148	FADS2	PFKFB4	TM7SF2
C3AR1	FADS3	PNCK	TNF
C6orf223	FAM127C	PNPLA3	WDR54
CCL3	FTSJ3	RENBP	
CCL3L1	GPNMB	RGS16	

Table S11– List of downregulated genes of shSF2-S vs Control-S analysis.

Gene Name	Gene Name	Gene Name	Gene Name
AAAS	CD9	MIF	SLC2A3
AACS	CYP19A1	NEU1	SLC43A1
ACSS2	EBP	NKD1	SLC7A11
ACY1	FADS2	NKG7	SLC7A7
ADAMTS1	FADS3	OLIG2	SPIB

ALDOC	FDPS	POFUT2	STT3A
APOC1	FTSJ3	PRAME	THBD
BCL2L14	HIST1H4E	PSMB9	THTPA
C10orf57	HIST1H4F	RENBP	TM7SF2
C11orf51	HMOX1	SBF2	TNR
C12orf10	IFI27	SCD	TRIM16L
C20orf20	LDHA	SF3B3	TUBA1A
C3AR1	MAML2	SFRS1	WDR54
CCL1	MEIS2	SLAMF8	
CD209	METTL7B	SLC2A14	

7.7 - ASE for the different analysis of the Human Exon 1.0 ST Arrays

Table S12– List of inclusion ASE observed in the Control-S vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ACHE	EIF4H	NFATC1	SLC25A23
AKAP1	EMR2	NR4A3	SLC2A6
ALAS1	ERLIN2	OXA1L	SSRP1
APEH	GBP6	PARVG	STAT5A
ATP13A2	GLUL	PKM2	STAT5B
ATP2B4	HAS3	PLCB2	TBCD
ATXN7L1	HELLS	PLEKHA4	TGM5
BAIAP2	IDH3B	PPARD	TNFRSF9
C1orf61	IL32	PSRC1	TNFSF10
CAMK2A	IL8	RBCK1	TNIP3
CCND3	IL8RB	RCAN1	UBAP2L
CD44	INSIG1	RCAN2	UBAP2L
CRIM1	IQSEC3	RET	VAPA
CTSH	IVNS1ABP	RGS8	VSNL1
DAZ3	KIF1A	RIN1	ZP3
EEF1A2	KLRD1	SERPINB12	
EEF1D	MED15	SETD8	
EIF4G3	MGLL	SHD	

Table S13– List of inclusion ASE observed in the shSF2-NS vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
A2M	HDLBP	PPIH	SCMH1
AGRN	IRF7	PTCRA	STAT5A
CYB5R2	MRPL13	PTDSS2	STAU1
FAM122B	PAK4	PTPRF	SYTL2

GPNMB	PNCK	RGS8	UBAP2L

Table S14– List of inclusion ASE observed in the shSF2-S vs Control-S analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ATG16L2	ESPN	LRP5L	SFXN1
C15orf26	FADS3	MAPK8IP3	SNPH
C4orf29	FAM62A	MARCH6	SNUPN
CCDC12	FAM69A	MPDZ	SON
CD1E	FDXR	NEDD4L	SRCAP
CDH26	GLTSCR2	NFYC	THOC2
CHMP6	GLUL	OR2H1	TIRAP
CHP	GORASP1	PEAR1	TNFRSF13B
CLEC4M	HDAC6	PI4KA	TNR
CNOT4	HDLBP	PIBF1	TRERF1
COL11A2	HERC2	PPIH	TRPC4
CSMD3	HSDL2	PRPF38B	USP15
DKKL1	IDI1	RP1L1	ZMYM2
EFNA1	LOC144983	SCMH1	ZNF501

Table S15– List of exclusion ASE observed in the Control-S vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ANKZF1	INSR	PAFAH1B1	SLC9A3R1
ARSG	KNTC1	PARD3	THOC2
ATAD3C	MAPKBP1	PDLIM5	THRA
BCL2L11	MFAP5	PHLDB1	TNFAIP2
BID	MFSD2	PMS2	TNIP1
C18orf22	MGLL	PNKD	TPM2
CD1E	MMP14	POU2F2	TRAF3IP2
DHX58	NARF	PRIC285	TRIP10
DISC1	NCOR2	PSPH	TTBK2
EFCAB1	NFKBID	PSRC1	TTC7A
FAM62B	NFKBIZ	RCSD1	ZIC5
FMNL3	NUP188	RGS18	ZNF217
HLA-E	NUP214	SH3BP5	

Table S16– List of exclusion ASE observed in the shSF2-NS vs Control-NS analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ABCE1	DHX58	OS9	TMEM134
ACADM	ENTPD6	PI4KB	TMEM9B
AHSA1	FAM49B	PLEKHA4	TMPRSS9
AKT1	GNAS	POLR1D	TRAIP

ALAS1	HMGA1	PVRL2	TXNDC11
BAIAP3	HNRNPD	RAB5C	UBAP2L
BCL2L13	IMPDH1	RPN2	VPS37A
CASP2	INSIG1	SLC37A2	VTI1A
CD1E	KIAA0515	SPPL3	YWHAB
CD9	MTMR3	SSR2	ZNF202
CD99L2	NMT1	ST3GAL3	

Table S17– List of exclusion ASE observed in the shSF2-S vs Control-S analysis.

Gene Name	Gene Name	Gene Name	Gene Name
ACAT2	HDGF	PNN	SMARCA4
ACTG2	HP1BP3	PRKDC	SPARCL1
ALDH16A1	IPO13	PRMT2	SUSD3
ARFGAP3	KLHL18	PSAT1	TFG
BRD7	LOC23117	REM2	TMEM161A
C21orf66	LRP10	RIMS1	TPM1
CCND3	NAPB	SERGEF	TXNDC11
CDC2L5	NOL14	SH3KBP1	USH1C
DAPL1	NRAP	SKIV2L2	WDR49
EIF4G3	PDXK	SLC44A1	ZNF138
ERMP1	PI4KB	SMAD5	

Table S18– List of the common genes identified in the ASF/SF2 CLIP-seq analysis (*Sanford et al.*, 2009) and the ASE observed in the shSF2-S vs Control-S analysis.

Common ASE of shSF2-S vs Control-S analysis and ASF/SF2 CLIP-seq (*Sanford et al.*, 2009)			
AATF	GOLGA8A	PI4KA	SLC15A4
ACTR2	GPI	PIBF1	SLC1A5
ACVR2B	GTPBP1	PLCB3	SLC2A1
ADAMTS1	H2AFX	PLEKHF2	SLC3A2
ADCY3	HDGF	PLK1	SLC43A1
ALKBH4	HDGF2	PNN	SMARCA4
AP2B1	HDLBP	PNPLA3	SMPD4
ARIH2	HMBS	POLQ	SNRPF
ATAD3C	HNRPDL	POLR1D	SNX15
ATP5J	HSPA4	PPFIA3	SRP68
ATP5L	IDI1	PPIE	SRRM2
BAG1	IGF2R	PPIH	TBC1D9B
BIRC5	IRAK1	PRAGMIN	TIRAP
BOLA1	KIAA0515	PRKAR1A	TLK1
BRD7	LAPTM4B	PRKCD	TMEM103
C12orf10	LDHA	PRKDC	TMEM120A
C18orf22	LIPT1	PRMT2	TMEM160
C4orf29	LONP1	PSAT1	TPM1
C9orf78	LRRC37A2	PSMC1	TRERF1
C9orf86	LYSMD1	PSRC1	TSPYL4
CANX	MCART1	QPRT	TTLL5

CCAR1	MICALL1	RBM3	TUBA1A
CDC2L5	MLL2	RCC1	TUBA1C
CDRT4	MLL4	REXO1	UBP1
CLGN	MLXIP	RNF157	UBQLN2
CLNS1A	MRTO4	RNPS1	WBSCR16
CLSTN1	MSH2	ROCK1	WDR42A
CNPY3	MYL6	RPN2	YEATS4
COG8	MYSM1	RPS21	YIPF6
COPS8	NBPF14	RRS1	ZBED4
EIF3CL	NDUFV3	RWDD3	ZC3H7B
EIF4B	NIP30	SAPS1	ZHX1
ERCC8	NUDC	SAPS2	ZKSCAN5
FAM127A	OS9	SAT2	ZNF138
FAM46B	PARP1	SEC24C	ZNF653
FDXR	PDXK	SF3B2	ZNF704
FUZ	PEA15	SFRS14	
GLUL	PFKP	SFRS16	

7.8 - Overexpression of SFRS1, SFRS3, IL-1β and IL-18.

7.8.1 - pENTR11 vectors.

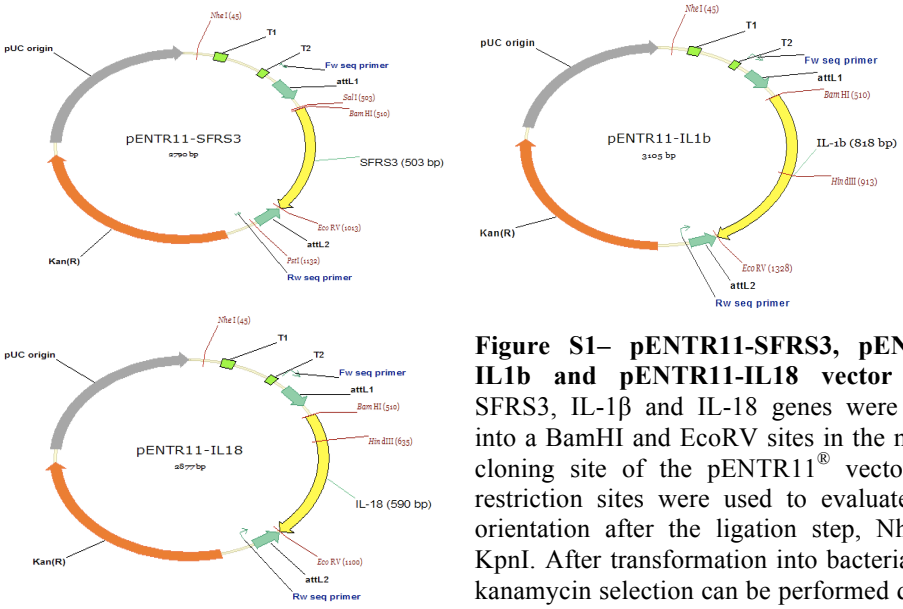


Figure S1– pENTR11-SFRS3, pENTR11-IL1b and pENTR11-IL18 vector maps. SFRS3, IL-1β and IL-18 genes were cloned into a BamHI and EcoRV sites in the multiple cloning site of the pENTR11® vector. Two restriction sites were used to evaluate insert orientation after the ligation step, NheI and KpnI. After transformation into bacterial cells, kanamycin selection can be performed due to a kanamycin resistance gene (Kan(R)) present in the final vector. Sequencing primers were designed to anneal in the outside of the multiple cloning site, therefore could be used to sequence all the constructs performed.

7.8.2 - pLenti6/V5-DEST vectors.

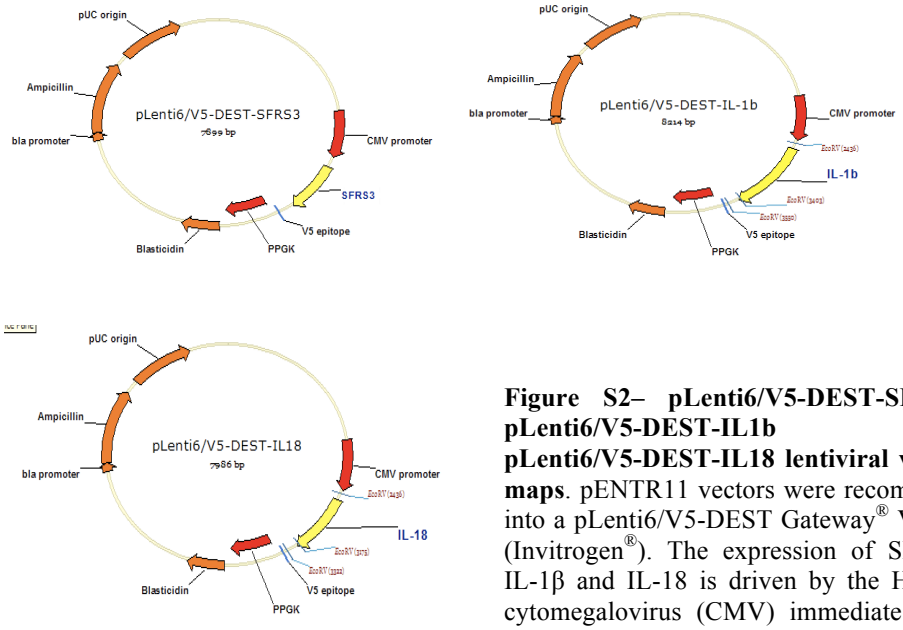


Figure S2– pLenti6/V5-DEST-SFRS3, pLenti6/V5-DEST-IL1b and pLenti6/V5-DEST-IL18 lentiviral vector maps. pENTR11 vectors were recombined into a pLenti6/V5-DEST Gateway® Vector (Invitrogen®). The expression of SFRS3, IL-1β and IL-18 is driven by the Human cytomegalovirus (CMV) immediate early promoter, which allows high-level and constitutive expression of the gene of interest (*Boshart et al.*, 1985). A PPGK promoter allows high-level expression of the Blasticidin resistance gene, allowing for transduced cells selection (*Kimura et al.*, 1994). Ampicillin resistance gene allows selection of the plasmid in *E. coli*, while pUC origin permits high-copy replication and maintenance in *E.coli* cells. Several restriction sites such as KpnI can be used to digest the plasmid and check for insert orientation and expression. Insert expression in mammalian cells can be visualized by the detection of the V5 epitope.

7.9 - Overexpression of IL-1β and/or IL-18 impact on IL-1β secretion after 24 hr *E.coli* challenge.

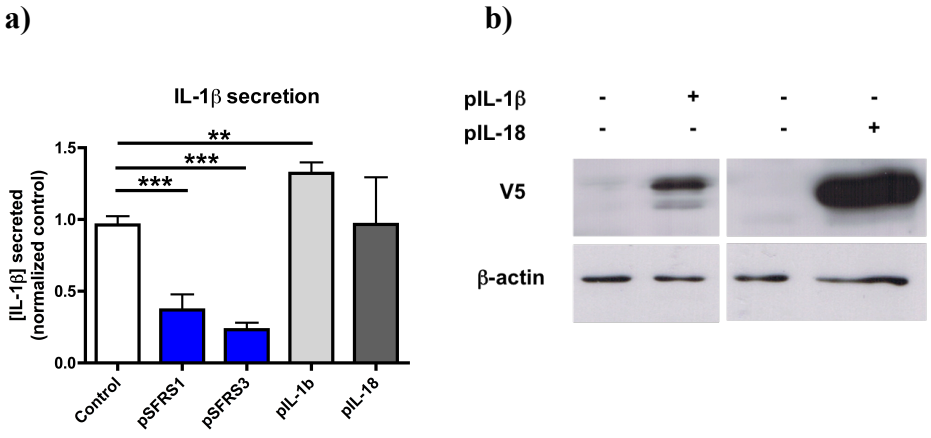
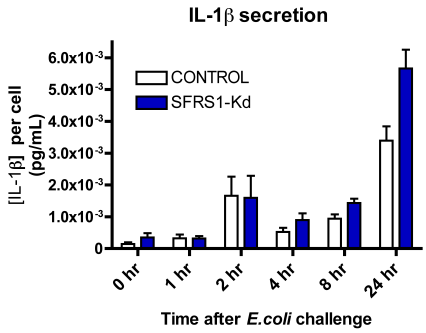


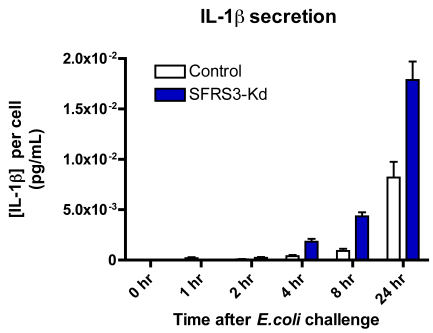
Figure S3- Overexpression of IL-1β and IL-18. Lentiviruses were used to overexpress IL-1β and IL-18 in THP-1 cells. After infection cells were challenged with *E.coli* and IL-1β concentration was measured in the supernatants by ELISA. (a) Overexpression of IL-1β increased IL-1β secretion after 24 hr *E.coli* challenge, as compared to control. In opposition, IL-18 overexpression did not show any impact on IL-1β secretion after *E.coli* challenge. (b) Western blot detection V5-epitope tagged IL-1β and IL-18 proteins. pSFRS1, pSFRS3, pIL-1β and pIL-18 are the lentivirus encoding the SFRS1, SFRS3 , IL-1β and IL-18 V5 tagged proteins. Lanes 1 and 3 are control non-nfected cells. Lanes 2 and 4 are cells overexpressing IL-1β and IL-18, respectively (t-student:**p<0.005, ***p<0.0001).

7.10 - IL-1β secretion kinetics upon SFRS1, SFRS3 or HMGB1 knockdown.

a)



b)



c)

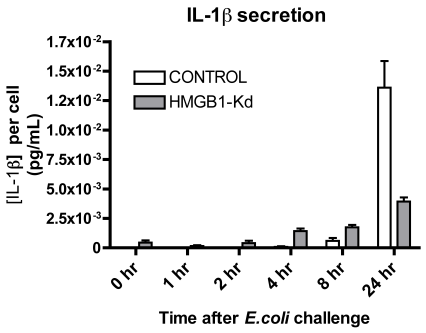


Figure S4– Kinetics of IL-1β secretion upon SFRS1,SFRS3 or HMGB1 knockdown. a,b) Increased IL-1β secretion, detected by ELISA, in cells where SFRS1 and SFRS3 were downregulated. Higher induction was found in SFRS3 knockdown cells, as compared with control and SFRS1 RNAi targeted cells. c) Decreased IL-1β secretion observed at 24 hr post *E.coli* challenge in cells where HMGB1 was previously downregulated.

7.11 – SFRS1 and SFRS3 expression upon *Mycobacterium tuberculosis* challenge

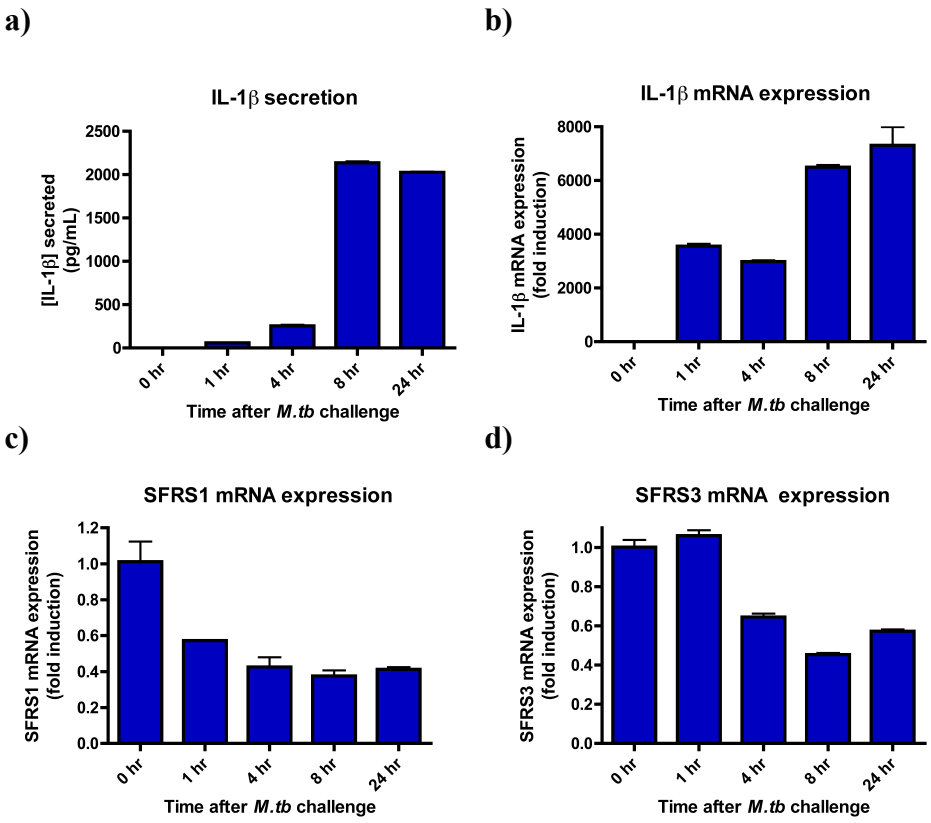
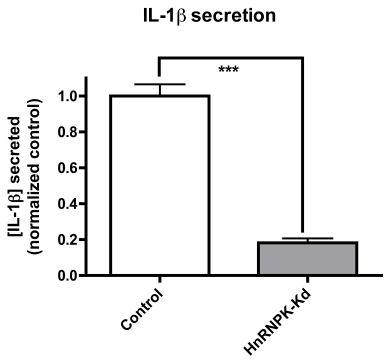


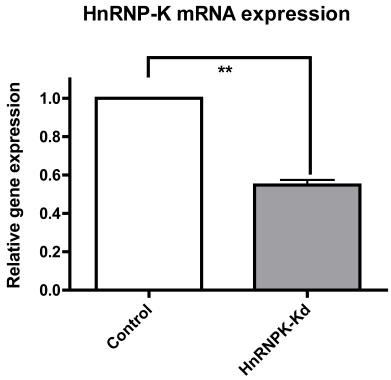
Figure S5 – *M.tb* impact on IL-1β secretion and IL-1β, SFRS1 and SFRS3 mRNA expression. 4% PFA fixed *M.tb* was used to challenge THP-1 cells at different time points. a,b) *M.tb* infection induced IL-1β mRNA expression and secretion. c,d) SFRS1 and SFRS3 mRNA expression is downregulated upon *M.tb* challenge.

7.12 - HnRNPK role in IL-1β secretion

a)



b)



c)

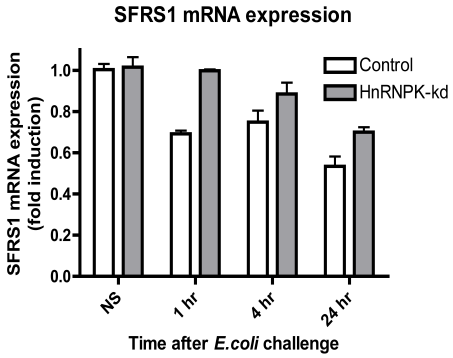


Figure S6 - HnRNPK role in IL-1β secretion. a) Knocking-down the expression of HnRNPK leads to decreased IL-1β secretion after 24 hr *E.coli* challenge. b) Validation of HnRNPK knockdown by qRT-PCR. c) SFRS1 expression is less downregulated upon *E.coli* challenge in cells previously knocked-down for the expression of HnRNPK.

7.13 – Expression of other SF Negative regulator candidates upon *E.coli* challenge

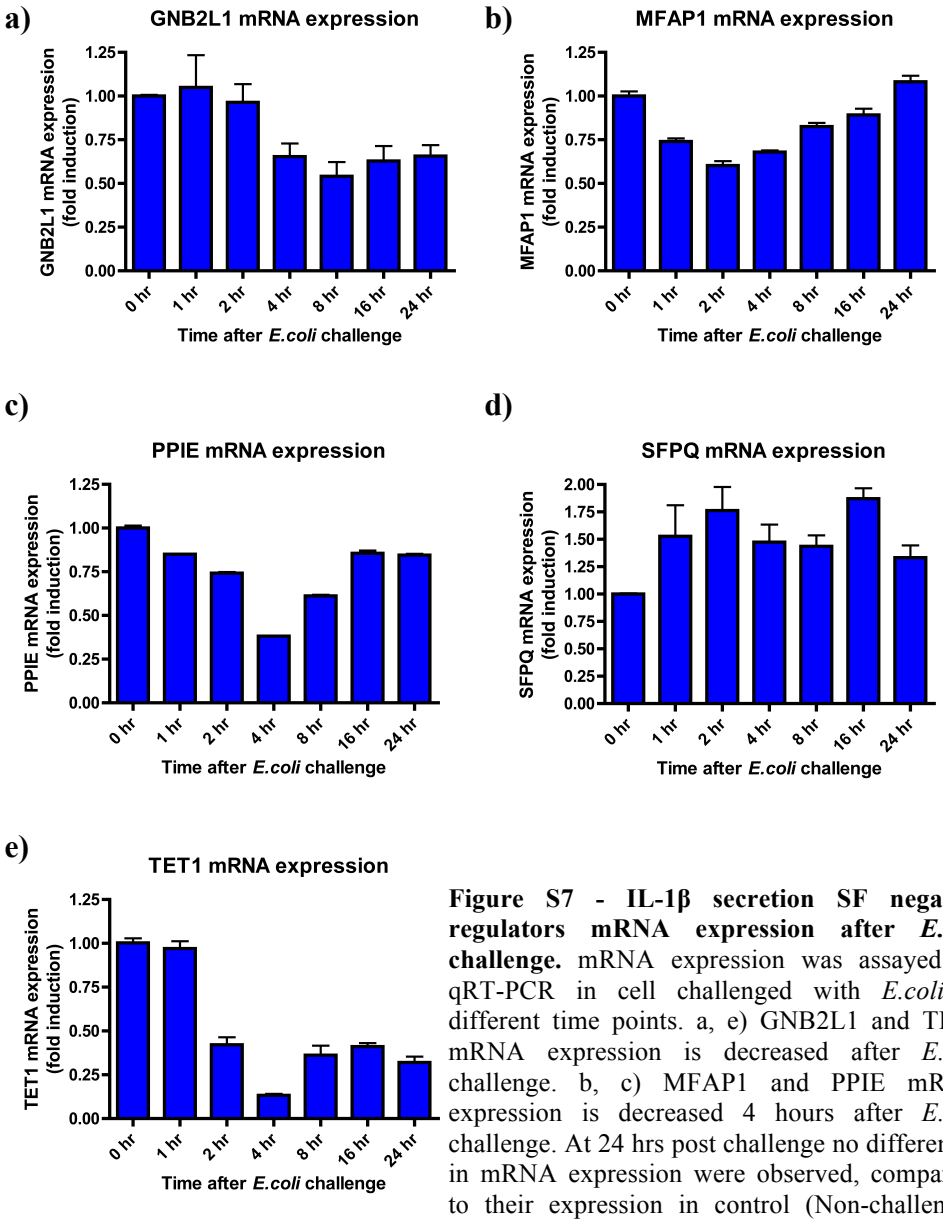
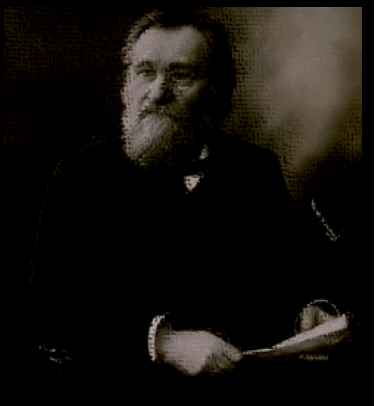


Figure S7 - IL-1β secretion SF negative regulators mRNA expression after *E.coli* challenge. mRNA expression was assayed by qRT-PCR in cell challenged with *E.coli* at different time points. a, e) GNB2L1 and TET1 mRNA expression is decreased after *E.coli* challenge. b, c) MFAP1 and PPIE mRNA expression is decreased 4 hours after *E.coli* challenge. At 24 hrs post challenge no differences in mRNA expression were observed, comparing to their expression in control (Non-challenged cells). d) SFPQ mRNA expression is slightly induced upon *E.coli* challenge.

"The experiment I have just outlined shows the first stage of inflammation in the animal world. Now inflammation as understood in man and the higher animals is a phenomenon that almost always results from the intervention of some pathogenic microbe..... Cure would come from the victory of the cells and immunity would be the sign of their acting sufficiently to prevent the microbial onslaught."

Metchnikoff, Nobel Lecture in 1908



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"A number of genes in higher organisms and in their viruses appear to be split... The cell produces a full RNA transcript of this DNA…and then appears to splice out the nonsense sequences before sending the RNA to the cytoplasm

…

a stretch of DNA in virus may produce more than one protein, depending on which way the primary transcript is spliced."

Francis Crick, 1979